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SMALL AND INTERMEDIATE CONDUCTANCE, CALCIUM-
ACTIVATED POTASSIUM CHANNELS AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of the filing date of USSN
5 60/026,451, filed on September 11, 1997; USSN 60/040,052, filed on March 7,
1997; and USSN 60/045,233, filed on April 17, 1997.

FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

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10 Health. The United States Government has certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to compositions relating to, and
methods for identifying, small conductance (SK) and intermediate conductance
(IK), calcium-activated potassium channels. The invention further provides a
15 method to assay for compounds that increase or decrease potassium ion flux
through calcium-activated potassium channels.

BACKGROUND OF THE INVENTION

Calcium-activated potassium currents are found in a wide variety of
animal cells such as nervous, muscular, glandular or epithelial tissue and from the
20 immune system. The channels regulating these currents open and allow the
escape of potassium as the internal calcium concentration increases. This
outward flow of potassium ions makes the interior of the cell more negative,
counteracting depolarizing voltages applied to the cell.

Two distinct classes of calcium-activated K⁺ channels (K_{ca} channels) have been described. Large conductance calcium-activated K⁺ channels (BK channels) are gated by the concerted actions of internal calcium ions and membrane potential, and have a unit conductance between 100 and 220 pS.

5 Small (SK) and intermediate (IK) conductance calcium-activated K⁺ channels are gated solely by internal calcium ions, with a unit conductance of 2-20 and 20-85 pS, respectively, and are more sensitive to calcium than are BK channels (for review see Latorre *et al.*, 1989, *Ann Rev Phys*, 51, 385-399.). In addition, each type of K_{ca} channel shows a distinct pharmacological profile. All three classes are

10 widely expressed, and their activity hyperpolarizes the membrane potential. Members of the BK (Atkinson *et al.*, 1991, *Science*, 253, 551-555.; Adelman *et al.*, 1992 *Neuron*, 9, 209-216.; Butler, 1993, *Science*, 261, 221-224) and SK (Kohler *et al.*, 1996, *Science*, 273, 1709-1714.) subfamilies have been cloned and expressed in heterologous cell types where they recapitulate the fundamental

15 properties of their native counterparts.

In vertebrate neurons action potentials are followed by an afterhyperpolarization (AHP) that may persist for several seconds and have profound consequences for the firing pattern of the neuron. Alterations in the AHP have been implicated in seizure activity (Alger *et al.*, *J. Physiol.* 399:191-205

20 (1988)) and learning and memory (de Jonge *et al.*, *Exp. Br. Res.* 80:456-462 (1990)). The AHP is composed of two prominent components, a fast component (fAHP) which mediates spike frequency at the onset of a burst, and a subsequent slow component (sAHP) which is responsible for spike-frequency adaptation (Nicoll, *Science* 241:545-551 (1988)).

25 Each component of the AHP is kinetically distinct and is due to activation of different calcium-activated potassium channels. Activation of large-conductance (100-200 pS), voltage- and calcium-activated potassium channels (BK channels) underlies the fAHP (Lancaster *et al.*, *J. Physiol.* 389:187-203 (1987); Viana *et al.*, *J. Neurophysiol.* 69:2150-2163 (1993)) which

30 develops rapidly (1-2 ms) and decays within tens of milliseconds. The channels underlying the sAHP are small conductance, calcium activated, potassium channels (SK channels) which differ from BK channels. being more calcium-sensitive, are not voltage-gated, and possessing a smaller unit conductance

(Lancaster *et al.*, *J. Neurosci.* 11:23-30 (1991); Sah, *J. Neurophysiol.* 74:1772-1776 (1995)).

5 The fAHP and the sAHP also differ in their pharmacology. The fAHP is blocked by low concentrations of external tetraethylammonium (TEA) and charybdotoxin (CTX), in accord with the pharmacology of the BK channels. Lancaster *et al.*, *J. Physiol.* 389:187-203 (1987); Viana *et al.*, *J. Neurophysiol.* 69:2150-2163 (1993); Butler *et al.*, *Science* 261:221-224 (1993). In contrast, the sAHP is insensitive to CTX, but fall into two classes regarding sensitivity to the bee venom peptide toxin, apamin. For example, in hippocampal pyramidal
10 neurons, the sAHP is insensitive to apamin (Lancaster *et al.*, *J. Neurophysiol.* 55:1268-1282 (1986)), while in hippocampal interneurons and vagal neurons it is blocked by nanomolar concentrations of the toxin (Sah, *J. Neurophysiol.* 74:1772-1776 (1995); Zhang *et al.*, *J. Physiol.* 488:661-672 (1995)).

15 In addition to its role in neuronal cells, non-voltage gated, apamin-sensitive potassium channels activated by submicromolar concentrations of calcium have also been described from peripheral cell types, including skeletal muscle (Blatz *et al.*, *Nature* 323:718-720 (1986)), gland cells (Tse *et al.*, *Science* 255:462-464 (1992); Park, *J. Physiol.* 481:555-570 (1994)) and T-lymphocytes (Grissmer *et al.*, *J. Gen. Physiol.* 99:63-84 (1992)).

20 For example, SK channels have been suggested to represent the apamin receptor found in muscle membrane of patients with myotonic muscular dystrophy. Renaud *et al.*, *Nature* 319:678-680 (1986)). Also, Grissmer *et al.* (*J. Gen. Physiol.* 99:63-84 (1992)) report that CTX insensitive, apamin sensitive calcium-activated potassium channels were identified in a human leukemic T cell
25 line and suggest that calcium-activated potassium channels play a supporting role during T-cell activation by sustaining dynamic patterns of calcium signaling. And in many cells, SK channels are activated as a result of neurotransmitter or hormone action. Haylett *et al.*, in *Potassium Channels: Structure, Classification, Function and Therapeutic Potential* (Cook, N.S., ed.), pp.71-95, John Wiley and
30 Sons, 1990). Intermediate channels play a role in the physiology of red blood cells.

Intermediate conductance, calcium activated potassium channels have been previously described in the literature by their electrophysiology. The

Gardos channel is opened by submicromolar concentrations of internal calcium and has a rectifying unit conductance, ranging from 50 pS at -120 mV to 13 pS at 120 mV (symmetrical 120 mM K⁺; Christophersen, 1991, *J. Membrane Biol.*, 119, 75-83.). It is blocked by charybdotoxin (CTX) but not the structurally related peptide iberiotoxin (IBX), both of which block BK channels (Brugnara *et al.*, 1995a, *J. Membr. Biol.*, 147, 71-82). Apamin, a potent blocker of certain native (Vincent *et al.*, 1975, *J. Biochem.*, 14, 2521.; Blatz and Magleby, 1986, *Nature*, 323, 718-720.) and cloned SK channels do not block IK channels (de-Allie *et al.*, 1996, *Br. J. Pharm.*, 117, 479-487). The Gardos channel is also blocked by some imidazole compounds, such as clotrimazole, but not ketoconazole (Brugnara *et al.*, 1993, *J. Clin. Invest.*, 92, 520-526). The electrophysiological and pharmacological properties of the Gardos channel show that it belongs to the IK subfamily of this invention.

IK channels have been described in a variety of other cell types.

Principle cells of the rat cortical collecting duct segregate different classes of K⁺ channels to the luminal and basolateral membranes. IK channels are present in the basolateral membrane where they promote the recirculation of K⁺ across this membrane, elevating the activity of the Na⁺ + K⁺-ATPase and thereby Na⁺ reabsorption into the blood (Hirsch and Schlatter, 1995, *Pflügers Arch. - Eur. J. Physiol.*, 449, 338-344.) IK channels have also been implicated in the microvasculature of the kidney where they may be responsible for the vasodilatory effects of bradykinin (Rapacon *et al.*, 1996). In brain capillary endothelial cells, IK channels are activated by endothelin, produced by neurons and glia, shunting excess K⁺ into the blood (Renterghem *et al.*, 1995, *J. Neurochem.*, 65, 1274-1281). Neutrophil granulocytes, mobile phagocytic cells which defend against microbial invaders, undergo a large depolarization subsequent to agonist stimulation, and IK channels have been implicated in repolarizing the stimulated granulocyte (Varnai *et al.*, 1993, *J. Physiol.*, 472, 373-390.). IK channels have also been identified in both resting and activated human T-lymphocytes. Grissmer *et al.* 1993, *J. Gen. Physiol.* 102, 601-630 reported that IK channels were blocked by low nanomolar concentrations of charybdotoxin, showed little or no voltage dependence, and were insensitive to apamin. This channel has also been identified in human erythrocytes, where it plays an important role in intracellular

volume homeostasis (Joiner, C.H., 1993, *Am. J. Physiol.* 264: C251-270 and in smooth muscle (Van Renterghem, C. *et al.* 1996, *J. Neurochemistry* 65,1274-1281.

Thus, it appears that SK and IK channels comprise a subfamily of calcium-activated potassium channels which play key physiological roles in many cell types. Accordingly, given the key role of SK and IK channels in a wide variety of physiological functions, what is needed in the art is the identification of novel SK and IK channel proteins and the nucleic acids encoding them. Additionally, what is needed are methods of identifying compounds which increase or decrease SK and IK channel currents for their use in the treatment or regulation of: learning and memory disorders, seizures, myotonic dystrophies, immune responses, and neurotransmitter or hormone secretions. The present invention provides these and other advantages.

SUMMARY OF THE INVENTION

In a first broad context, this invention provides for novel proteins and their corresponding nucleic acids where the proteins are defined as monomers of calcium activated potassium ion channels. The monomers have a molecular weight of between 40 and 80 kDa and have units of conductance of between 2 and 80 pS when the monomer is in the polymeric form as expressed in *Xenopus* oocytes. In addition, the monomer specifically binds to antibodies generated against SEQ ID NO:30 or 42.

In another aspect, the present invention relates to an isolated nucleic acid encoding at least 15 contiguous amino acids of a calcium-activated potassium channel protein. The SK channel protein has a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47 and conservatively modified variants of SEQ ID NOS:1, 2, 3, 4, 19, 20, 32, 43 or 47.

In some embodiments, the isolated nucleic acid encodes a calcium-activated potassium channel protein having a conductance of at least 2pS when expressed in a *Xenopus* oocyte, a molecular weight of between 40 and 100 kilodaltons (kd), and selectively hybridizes, under stringent hybridization

conditions, with SK or IK encoding nucleic acid such as SEQ ID NO:13 in a human genomic library or SEQ ID NO:14 in a rat genomic library. In other embodiments, the isolated nucleic acid encoding the calcium-activated potassium channel protein encodes a protein having a sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47. In preferred embodiments the nucleic acid has a sequence selected from the group consisting of: SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:31, SEQ ID NO:44, and SEQ ID NO:48.

In another aspect, the present invention relates to an isolated calcium-activated potassium channel protein having at least 15 contiguous amino acids of a sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47, and conservatively modified variants of SEQ ID NOS:1, 2, 3, 4, 19, 20, 32, 43, or 47, wherein the variant specifically reacts, under immunologically reactive conditions, with an antibody reactive to a protein selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47.

In a broad embodiment, the calcium-activated potassium channel protein is defined as having a conductance of at least 2pS and a molecular weight of between 40 and 100 Kd. In other embodiments, the calcium-activated potassium channel protein has an amino acid sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47.

In another aspect, the present invention is directed to an antibody specifically reactive, under immunologically reactive conditions, to a calcium-activated potassium channel protein, where the protein has a sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47. In preferred embodiments, the antibody is limited to a monoclonal antibody.

In yet another aspect, the present invention relates to an expression vector comprising a nucleic acid encoding a monomer of a calcium-activated potassium channel where the monomer has a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47, and conservatively modified variants of SEQ ID NOS:1, 2, 3, 4, 19, 20, 32, 43, or 47 wherein the modified variant is a protein having a conductance of at least 2pS when expressed in a *Xenopus* oocyte, a molecular weight of between 40 and 100 kd, and specifically reacts, under immunologically reactive conditions, with an antibody reactive to a full-length protein selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47.

In another aspect, the present invention relates to a host cell transfected with a vector comprising a nucleic acid encoding a monomer of a calcium-activated potassium channel protein where the protein has a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, SEQ ID NO:47, and conservatively modified variants of SEQ ID NO:1, 2, 3, 4, 19, 20, 32, 43 or 47 wherein the modified variant is a protein having a conductance of at least 2pS when expressed in a *Xenopus* oocyte, a molecular weight of between 40 and 100 Kd, and specifically reacts, under immunologically reactive conditions, with an antibody reactive to a full-length protein selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47. Typically, the host cell is cultured under conditions permitting expression of the nucleic acid encoding the calcium-activated potassium channel protein.

In yet a further aspect, the present invention relates to an isolated nucleic acid sequence of at least 15 nucleotides in length which specifically hybridizes, under stringent conditions, to a nucleic acid encoding a calcium-activated potassium channel protein, where the protein is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47.

In an additional aspect, the present invention is directed to a method for detecting the presence of a calcium-activated potassium channel protein in a biological sample. The method comprises contacting the biological sample with an antibody, wherein the antibody specifically reacts, under immunologically reactive conditions, to an calcium-activated potassium channel protein having a sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47 and allowing the antibody to bind to the protein under immunologically reactive conditions, wherein detection of the bound antibody indicates the presence of the channel protein.

In yet another aspect, the present invention provides a method for detecting the presence, in a biological sample, of a nucleic acid sequence encoding a calcium-activated potassium channel protein of at least 25 amino acids in length. The method comprises contacting the biological sample, under stringent hybridization conditions, with a nucleic acid probe comprising a nucleic acid segment that selectively hybridizes to a nucleic acid encoding the channel protein having a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47; allowing the nucleic acid encoding the channel protein to selectively hybridize to the probe to form a hybridization complex, wherein detection of the hybridization complex is an indication of the presence of the nucleic acid sequence in the sample. In some embodiments, the hybridization conditions are moderate stringency hybridization conditions. In another embodiment, the calcium activated channel protein is at least 400 amino acid residues in length and when expressed in oocytes has a conductance of at least 2 pS. In a further embodiment, the nucleic acid probes comprises at least 250 contiguous nucleotides encoding a subsequence within the small or intermediate calcium-activated potassium channel protein core region.

In a further aspect, the present invention relates to an isolated calcium-activated potassium channel encoded by a nucleic acid amplified by primers which selectively hybridize, under stringent hybridization conditions, to the same nucleic acid sequence as primers selected from the group consisting of: for hSK1, SEQ ID NO:5 and SEQ ID NO:6; for rSK2 SEQ ID NO:7 and SEQ ID NO:8;

for endogenous rSK3, SEQ ID NO:9 and SEQ ID NO:10; for rSK1, SEQ ID NO:11 and SEQ ID NO:12; for hSK2, SEQ ID NO:23 and SEQ ID NO:24; for hSK3, SEQ ID NO:25 and SEQ ID NO:26; and for hIK the following primer pairs will amplify a probe that is selective for identifying hIK1 from a human genomic or cDNA library:

5' GCCGTGCGTGCAGGATTTAGG 3' (SEQ ID NO:34) and
5'CCAGAGGCCAAGCGTGAGGCC 3' (SEQ ID NO:35) yielding a probe of about 270 bases or 5' TCCAAGATGCACATGATCCTG 3' (SEQ ID NO:36) and 5' GGACTGCTGGCTGGGTTCTGG 3' (SEQ ID NO:37) yielding a probe of about 165 bases. For amplification of a full length hIK1 either of the following two primer pairs will work: 5' ATGGGCGGGGATCTGGTGCTTG 3' (SEQ ID NO:38) and 5' CTACTTGGACTGCTGGCTGGGTTC 3' (SEQ ID NO:39) or 5' ATGGGCGGGGATCTGGTGCTTG 3' (includes codon of initiator methionine) (SEQ ID NO:40) and 5' GGGTCCAGCTACTTGGACTGCTG 3' (includes stop codon for end of translation) (SEQ ID NO:41).

In yet another aspect, the present invention relates to a method of identifying a compound which increases or decreases the potassium ion flux through a small or intermediate conductance, calcium-activated potassium channel, with the proviso that the compound is not clotrimazole. The method comprises the steps of contacting the compound with a eukaryotic host cell in which has been expressed a nucleic acid encoding a calcium-activated potassium channel having a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, SEQ ID NO:47 and conservatively modified variants thereof, wherein said conservatively modified variant specifically binds to antibodies specifically reactive with an antigen having an amino acid sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47, have a conductance of at least 2pS, and a molecular weight between 40 and 100 kilodaltons; and determining the increased or decreased flux of potassium ions through said channel. In preferred embodiments, the increased or decreased flux of potassium ions is determined by measuring the electrical current or flux of ions, or indirectly the change in voltage induced by the change in current or flux of ions, across the cell membrane of said eukaryotic host cell.

In a particularly preferred embodiment, the channel protein has a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47. In another preferred embodiment, the channel protein is recombinant.

5 In a further aspect, the present invention relates to an isolated eukaryotic nucleic acid encoding a calcium-activated potassium channel protein of at least 400 amino acid residues in length, wherein the calcium-activated channel protein comprises an amino acid sequence having at least 55 to 60% similarity over the length of a core region of a protein selected from the group consisting of: 10 SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47 and wherein the channel protein has a conductance of at least 2pS. In some embodiments, the present invention is directed to the protein encoded by the aforementioned 15 isolated eukaryotic nucleic acid. In other embodiments, the isolated nucleic acid encoding the calcium-activated channel protein has at least 85% sequence similarity over a comparison window of 20 contiguous amino acid residues within the core region.

In a further aspect, the present invention is directed to a vector 20 comprising an isolated eukaryotic nucleic acid encoding a calcium-activated potassium channel protein of at least 400 amino acid residues in length, wherein the channel protein comprises an amino acid sequence having at least 55% similarity over the length of a core region of a protein selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID 25 NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47, and wherein the channel protein has a conductance of at least 2pS. Typically, the vector is transfected into a host cell which is cultured under conditions permitting expression of the isolated eukaryotic nucleic acid encoding the channel protein.

30 In a further aspect, present invention is directed to a method of identifying a compound that increases or decreases the potassium ion flux through a calcium-activated potassium channel. The methods comprises the steps of contacting the compound with a eukaryotic host cell in which has been expressed a calcium-activated potassium channel protein of at least 400 amino

acid residues in length, wherein the channel protein has an amino acid sequence having at least 55% similarity over the length of a core region of a protein selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43, and SEQ ID NO:47, and wherein the channel protein has a conductance of at least 2pS; and determining the increased or decreased flux of potassium ions through the channel protein. In some embodiments the increased or decreased flux of potassium ions is determined by measuring the electrical current across the cell membrane of the eukaryotic host cell.

In another aspect, the present invention provides in a computer system a method of screening for mutations of SK and IK genes, the method comprising the steps of: (i) receiving input of a first nucleic acid sequence encoding a calcium-activated channel protein having a sequence selected from the group consisting of SEQ ID NOS:1, 2, 3, 4, 19, 20, 32, 43, 47 and conservatively modified versions thereof; (ii) comparing the first nucleic acid sequence with a second nucleic acid sequence having substantial identity to the first nucleic acid sequence; and (iii) identifying nucleotide differences between the first and second nucleic acid sequences. In one embodiment, the second nucleic acid sequence is associated with a disease state.

In another aspect, the invention provides in a computer system, a method for identifying a three-dimensional structure of SK and IK proteins, the method comprising the steps of: (i) receiving input of an amino acid sequence of a calcium-activated channel protein or a nucleotide sequence of a gene encoding the protein, the protein having an amino acid sequence selected from the group consisting of SEQ ID NOS:1, 2, 3, 4, 19, 20, 32, 43, 47, and conservatively modified versions thereof; and (ii) generating a three-dimensional structure of the protein encoded by the amino acid sequence. In one embodiment, the amino acid sequence is a primary structure and the generating step includes the steps of forming a secondary structure from the primary structure using energy terms encoded by the primary structure and forming a tertiary structure from the secondary structure using energy terms encoded by said secondary structure. In another embodiment, the generating step includes the step of forming a quaternary structure from the tertiary structure using anisotropy terms encoded

by the tertiary structure. In another embodiment, the method further comprises the step of identifying regions of the three-dimensional structure of the protein that bind to ligands and using the regions to identify ligands that bind to the protein.

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention provides novel isolated, small conductance, calcium-activated potassium (SK) channels, intermediate conductance, calcium-activated potassium (IK) channels (collectively, "calcium-activated potassium channels"), and isolated nucleic acids encoding SK and IK channels (i.e., SK and IK channel nucleic acids). The distribution, function, and pharmacology define
10 these new classes of channels as SK or IK channels.

 Expression of isolated SK or IK channel protein encoding nucleic acids in a host cell provides a composition which can be used to identify compounds that increase or decrease potassium ion flux through small conductance, calcium-activated potassium (SK) channels or intermediate
15 conductance, calcium-activated potassium (IK) channels, respectively. Since SK channels underlie the slow component of the afterhyperpolarization (sAHP) of neurons, alteration of neuronal sAHP provides a means to inhibit epileptic seizures or modulate learning or memory disorders.

 Calcium activated, SK channels are also implicated in T-cell
20 activation. Thus, increasing or decreasing SK channel currents provides a means to inhibit or potentiate the immune response. Moreover, SK channels are associated with hormone and neurotransmitter secretions. Accordingly, altering SK channel currents provides a means to regulate cellular or glandular secretions and thereby treat imbalances thereof.

25 Calcium activated intermediate channels (IK) are also believed to play an important physiological role particularly in peripheral tissues. For example, intermediate channels are reported in red blood cells, and, in part, contribute to cell dehydration, a process that is exacerbated in sickle cell anemia.

 The invention also relates to subsequences of isolated small
30 conductance and intermediate conductance, calcium-activated potassium channels and for isolated nucleic acids encoding SK and IK channel proteins.

Isolated nucleic acids coding for SK or IK channel proteins provide utility as probes for identification of aberrant transcription products or increased or decreased transcription levels of genes coding for SK or IK channels. Assaying for increased or decreased transcription can be used in drug screening protocols.

- 5 Likewise, SK or IK channel proteins can be used as immunogens to generate antibodies for use in immunodiagnostic assays of increased or decreased expression of calcium-activated potassium channels in drug screening assays.

Definitions

- 10 Units, prefixes, and symbols may be denoted in their SI accepted form. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. Numeric ranges are inclusive of the numbers defining the range. The terms defined below are more fully defined by reference to the specification as a whole.

- 15 The terms "nucleic acid" "probe", or "primer" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence
20 includes the perfect complementary sequence thereof. Eukaryotic nucleic acids are nucleic acids from eukaryotic cells, preferably cells of multicellular eukaryotes.

- The term "recombinant" when used with reference to a cell, or protein, nucleic acid, or vector, includes reference to a cell, protein, or nucleic acid, or vector, that has been modified by the introduction of a heterologous
25 nucleic acid or the alteration of a native nucleic acid to a form not native to that cell, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes and proteins that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

- 30 The term "subsequence" in the context of a referenced nucleic acid sequence includes reference to a contiguous sequence from the nucleic acid having fewer nucleotides in length than the referenced nucleic acid. In the

context of a referenced protein, polypeptide, or peptide sequence (collectively, "protein"), "subsequence" refers to a contiguous sequence from the referenced protein having fewer amino acids than the referenced protein.

5 The terms "identical" or "sequence identity" in the context of two nucleic acid or polypeptide sequences includes reference to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid
10 residues are substituted for other amino acid residues with similar chemical properties (e.g. charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well-known to those of skill in the art. Typically this involves scoring a
15 conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., according to the algorithm of Meyers and Miller, *Computer Applic. Biol. Sci.*, 4: 11-17 (1988) e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA).

25 A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are
30 well-known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2: 482; by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443; by the search for similarity method of

Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444; by computerized implementations of these algorithms (including, but not limited to CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, California, GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics
5 Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wisconsin, USA); the CLUSTAL program is well described by Higgins and Sharp (1988) *Gene*, 73: 237-244 and Higgins and Sharp (1989) *CABIOS* 5: 151-153; Corpet, *et al.* (1988) *Nucleic Acids Research* 16, 10881-90; Huang, *et al.* (1992) *Computer Applications in the Biosciences* 8, 155-65, and Pearson, *et al.* (1994)
10 *Methods in Molecular Biology* 24, 307-31. Alignment is also often performed by inspection and manual alignment.

The terms "substantial identity" or "similarity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 60% sequence identity, preferably at least 80%, more preferably at least 90% and
15 most preferably at least 95%, compared to a reference sequence using the programs described above (preferably BLAST) using standard parameters. One indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

Another indication that two nucleic acid sequences have
20 substantially identity is that the two molecules hybridize to each other under "moderate stringency hybridization conditions" (or "moderate conditions"). Exemplary "moderate stringency hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X
25 SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Nucleic acids which do not hybridize to each other under moderate stringency hybridization conditions are still substantially identical if the polypeptides which
30 they encode are substantially identical. This occurs, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

The terms "substantial identity" or "similarity" in the context of a peptide indicates that a peptide comprises a sequence with at least 60% sequence identity to a reference sequence, usually at least 70%, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Generally, similarity is determined using a comparison window having a length of any number from 20 contiguous positions to the number of residues in the full-length core region sequence (i.e., the region of optimal alignment with rSK2 from amino acid residue 135 to 462), where the comparison window is within the core sequence.

The terms "oligonucleotide" or "polynucleotide" probes include reference to both double stranded and single stranded DNA or RNA. The terms also refer to synthetically or recombinantly derived sequences essentially free of non-nucleic acid contamination.

As used herein, "contact" or "contacting" means to place in direct physical association.

"Biological sample" as used herein is a sample of biological tissue or fluid that contains an IK and/or SK channel protein or nucleic acid encoding the corresponding IK and/or SK channel protein. Such samples include, but are not limited to, sputum, amniotic fluid, blood, blood cells (e.g., white cells), or tissue. Biological samples may also include sections of tissues such as frozen sections taken for histological purposes. Examples of biological samples include a cell sample from nervous, muscular, glandular or epithelial tissue or from the immune system (e.g., T cells). A biological sample is typically obtained from a eukaryotic organism, preferably a multicellular eukaryotes such as insect, protozoa, birds, fish, reptiles, and preferably a mammal such as rat, mice, cow, dog, guinea pig, or rabbit, and most preferably a primate such as macaques, chimpanzees, or humans.

The term "antibody" also includes antigen binding forms of antibodies (e.g., Fab, F(ab)₂). The term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind and recognize an analyte (antigen). The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies exist e.g., as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab')₂, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The F(ab')₂ may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the F(ab')₂ dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (see, *Fundamental Immunology*, Third Edition, W.E. Paul, ed., Raven Press, N.Y. 1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments such as single chain Fv, chimeric antibodies (i.e., comprising constant and variable regions from different species), humanized antibodies (i.e., comprising a complementarity determining region (CDR) from a non-human source) and heteroconjugate antibodies (e.g., bispecific antibodies).

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

5 "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences.

10 Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such

15 nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a

20 functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid

25 or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art.

The following six groups each contain amino acids that are

30 conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);

- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

See also, Creighton (1984) Proteins W.H. Freeman and Company.

5 The terms "biologically pure" or "isolated" refer to material which is substantially or essentially free from components which normally accompany or interact with it as found in its naturally occurring environment. The isolated material optionally comprises material not found with the material in its natural environment.

10 The phrase "encodes a protein which could be encoded by a nucleic acid that selectively hybridizes under moderate stringency hybridization conditions to a sequence selected from the group consisting of:" in the context of nucleic acids refers to those nucleic acids encoding naturally occurring proteins or derivatives of natural proteins, but which are deliberately modified or engineered to no longer hybridize to the protein of natural origin under the stated conditions.

15 An "expression vector" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements which permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment.

20 Typically, the expression vector includes a nucleic acid to be transcribed, and a promoter.

 The phrase "functional effects" in the context of assays for testing compounds affecting the channel includes the determination of any parameter that is indirectly or directly under the influence of the channel. It includes changes in

25 ion flux and membrane potential but also includes other physiologic effects such as increases or decreases of transcription or hormone release.

 By "selectively hybridizing" or "selective hybridization" or "selectively hybridizes" is meant hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably

30 greater degree than its hybridization to non-target nucleic acid sequences and/or to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences have at least 80% sequence identity, preferably 90% sequence identity, and most preferably 100% sequence identity (i.e., complementary) with

each other. "Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not
5 comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the
10 result by 100 to yield the percentage of sequence identity.

The terms "stringent conditions" or "stringent hybridization conditions" refer to conditions under which a probe will hybridize to its target sequence, to a detectably greater degree than other sequences. Stringent conditions are sequence-dependent and will be different in different
15 circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched
20 probe. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also
25 be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 2X SSC at 50°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60°C.

30 "Stringent hybridization conditions" or "stringent conditions" in the context of nucleic acid hybridization assay formats are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in*

Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences
5 hybridize specifically at higher temperatures.

By "hybridization complex" is meant a duplex nucleic acid sequence formed by selective hybridization of two single-stranded nucleic acid sequences with each other.

10 By "host cell" is meant a cell which contains an expression vector and supports the replication or expression of the expression vector. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells.

15 By "conductance" is meant electrical conductance. Electrical conductance is conveniently measured in Siemens ($1/\text{ohm} = \text{mho}$). Unitary conductance is determined by measuring single channel currents using a patch clamp protocol under conditions set forth in Example 6 (i.e., in an oocyte) using a symmetrical potassium ion concentration of 120 mM. See generally, Hille, B.,
20 *Ionic Channels of Excitable Membranes*, 2nd ed., Sinauer Assoc., Sunderland, MA. In the context of the present invention, "conductance" refers to the unitary electrical conductance of a single homomeric protein of the referenced SK or IK channel protein.

25 By "when expressed in an oocyte leads to formation of an SK channel" includes reference to expression of a referenced SK protein in which a plurality of the referenced SK proteins are assembled to form, by themselves or in conjunction with other endogenous *Xenopus* oocyte molecules, an SK channel. Expression within a *Xenopus* oocyte is disclosed in the Examples provided herein, e.g., Example 3.

30 By "when expressed in an oocyte leads to formation of a calcium-activated potassium channel" includes reference to expression of a referenced IK and/or SK protein in which a plurality of the referenced IK and/or SK proteins are assembled to form, by themselves or in conjunction with other endogenous *Xenopus* oocyte molecules, a calcium-activated potassium channel. Expression

within a *Xenopus* oocyte is disclosed in the Examples provided herein, e.g., Example 3.

By "immunologically reactive conditions" is meant conditions which allow an antibody, generated to a particular epitope, to bind to that epitope to a detectably greater degree than the antibody binds to substantially all other epitopes. Immunologically reactive conditions are dependent upon the format of the antibody binding reaction and typically are those utilized in immunoassay protocols. See Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions.

By "antibody reactive to a protein" is meant the protein is "specifically immunoreactive with an antibody."

The phrase "specifically immunoreactive with an antibody", or "specifically binds to an antibody" when referring to a protein or peptide, refers to a binding reaction between an antibody and a protein having an epitope recognized by the antigen binding site of the antibody. This binding reaction is determinative of the presence of a protein having the recognized epitope amongst the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a protein having the recognized epitope and bind, if at all, to a detectably lesser degree to other proteins lacking the epitope which are present in the sample.

Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, antibodies raised to the calcium activated potassium channel protein with the amino acid sequence depicted in SEQ ID NO:1, 2, 3, 4, 19, 20, 30, 32, 43, and 47 can be selected from to obtain antibodies specifically immunoreactive with small and/or intermediate calcium activated potassium channel proteins and not with other proteins. The proteins used as immunogens can be in native conformation or denatured so as to provide a linear epitope.

A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988)

Antibodies. A Laboratory Manual, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

By "transfected" is meant the introduction of a nucleic acid into a eukaryotic cell where the nucleic acid may be incorporated into the genome of the cell (i.e., chromosome, plasmid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA). The transfection can be *in vivo* or *ex vivo*. "Ex vivo" means outside the body of the organism from which a cell or cells is obtained or from which a cell line is isolated. *Ex vivo* transfection is preferably followed by re-infusion of the cells back into the organism. In contrast, by "*in vivo*" is meant within the body of the organism from which the cell was obtained or from which a cell line is isolated.

By "antigen" is meant a substance to which an antibody can be generated and to which the antibody is specifically immunoreactive with. An antibody immunologically reactive with a particular antigen can be generated *in vivo* or by recombinant methods such as selection of libraries of recombinant antibodies in phage or similar vectors. See, e.g., Huse *et al.* (1989) *Science* 246:1275-1281; and Ward, *et al.* (1989) *Nature* 341:544-546; and Vaughan *et al.* (1996) *Nature Biotechnology*, 14:309-314.

By "encoding" or "encoded", with respect to a specified nucleic acid, is meant comprising the information for translation into the specified protein. The information is specified by the use of codons. Typically, the amino acid sequence is encoded by the nucleic acid using the "universal" genetic code. However, variants of the universal code, such as is present in some plant, animal, and fungal mitochondria, the bacterium *Mycoplasma capricolum* (*Proc. Natl. Acad. Sci.*, 82:2306-2309 (1985), or the ciliate *Macronucleus*, may be used when the nucleic acid is expressed using these organisms.

By "contiguous amino acids from" in the context of a specified number of amino acid residues from a specified sequence, is meant a sequence of amino acids of the specified number from within the specified reference sequence which has the identical order of amino acids each of which is directly adjacent to the same amino acids as in the reference sequence.

By "small conductance, calcium activated potassium channel" or "SK channel" is meant a membrane channel which is not voltage-gated, activated by calcium from about 30 nM to 10 μ M, and has a unitary conductance of from about 2 to 60 pS, often 2 to 25 pS, when measured under a symmetrical potassium concentration of 120 mM using the conditions specified in Example 6. An SK channel comprises multiple SK channel proteins as subunits, typically four SK channel proteins (e.g., full length or substantially full length SK channel proteins).

By "small conductance, calcium-activated channel protein" or "SK channel protein" is meant a peptide of at least 10 contiguous amino acids in length from an amino acid sequence which makes up an SK channel. These proteins, when full length, serve as monomers of the SK channel. Thus, an SK channel protein can have the functional characteristics to form a heteromeric or homomeric protein with the functional characteristics of an SK channel, or be a peptide fragment thereof. For example, both N-terminal extended rsk3 (SEQ ID NO:43 and truncated rsk3 (SEQ ID NO:3) demonstrate virtually identical functional characteristics.

By "intermediate conductance, calcium-activated potassium channel" or "IK channel" is meant a membrane channel which is not voltage-gated, activated by calcium from about 30 nM to 10 μ M, and has in its broadest context a unitary inward conductance of from about 20 to 80 pS, but more likely 30 to 70 pS, 40 to 60 pS, or most preferably about 35 to 40 pS when measured under a symmetrical potassium concentration of 120 mM using the conditions specified in Example 6. An IK channel comprises multiple IK channel proteins as subunits, typically four IK channel proteins (e.g., full length or substantially full length IK channel proteins).

By "intermediate conductance, calcium-activated channel protein" or "IK channel protein" is meant a peptide of at least 10 contiguous amino acids in length from an amino acid sequence which makes up an IK channel. These proteins, when full length, serve as monomers of the IK channel. Thus, an IK channel protein can have the functional characteristics to form a heteromeric or homomeric protein with the functional characteristics of an IK channel, or be a peptide fragment thereof.

By "calcium-activated potassium channel" means a small conductance, calcium-activated potassium (SK) channel, and an intermediate conductance, calcium-activated potassium (IK) channel.

5 The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

10 By "specifically reacts" or "specifically reactive" is meant a reaction of the specificity exhibited by that between an antibody and a protein which "specifically binds" with that antibody.

By "human genomic library" is meant a collection of isolated DNA molecules which substantially represent the entire genome of a human. Construction of genomic libraries is taught in standard molecular biology references such as Berger and Kimmel, *Guide to Molecular Cloning Techniques*, 15 *Methods in Enzymology* volume 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3; and *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel). 20

By "amplified" is meant the construction of multiple copies of a nucleic acid sequence or multiple copies complementary to the nucleic acid sequence using at least one of the nucleic acid sequences as a template. Amplification systems include the polymerase chain reaction (PCR) system, ligase 25 chain reaction (LCR) system, nucleic acid sequence based amplification (NASBA, Cangene, Mississauga, Ontario), Q-Beta Replicase systems, transcription-based amplification system (TAS), and strand displacement amplification (SDA). See, e.g., *Diagnostic Molecular Microbiology: Principles and Applications*, Ed. D. H. Persing *et al.*, American Society for Microbiology, Washington, D.C.

30 The term "residue" or "amino acid residue" or "amino acid" as used herein refers to an amino acid that is incorporated into a protein, polypeptide, or peptide (collectively "peptide"). The amino acid may be a naturally occurring amino acid and, unless otherwise limited, may encompass known analogs of

natural amino acids that can function in a similar manner as naturally occurring amino acids.

By "segment of nucleic acid" is meant a nucleic acid sequence of any one of from 15 to about 1500 nucleotides, or nucleotide analogs, in length or concatamers of such sequence.

By "determining the functional effect" is meant examining the effect of a compound that increases or decreases potassium ion flux on a cell or cell membrane in terms of cell and cell membrane function. Preferably, the term refers to the functional effect of the compound on SK and IK channel activity, e.g., changes in conductance, voltage gating and the like.

Small and Intermediate Conductance, Calcium-Activated Potassium Channel Proteins

The present invention provides intermediate conductance, calcium-activated (IK) potassium channel proteins, and small conductance, calcium-activated (SK) channel proteins (collectively, "calcium-activated potassium channels"). The isolated small conductance, calcium-activated (SK) channel proteins of the present invention comprise at least N amino acids from any one of the sequences selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:43, and SEQ ID NO:47, and conservatively modified variants thereof, where N is any one of the integers selected from the group consisting of from 10 to 600 and the sequence is unique to the protein of origin.

Similarly, the isolated intermediate conductance, calcium-activated (IK) channel proteins of the present invention comprise at least N amino acids from SEQ ID NO:32 and conservatively modified variants thereof, where N is any one of the integers selected from the group consisting of from 10 to 600 and the sequence is unique to the protein of origin.

Typically, the calcium-activated potassium channel proteins and specific peptides are at least 15, 25, 35, or 50 amino acids in length, more preferably at least 100, 200, 300, 400, or 500 amino acids in length, and most preferably the full length of SEQ ID NOS:1, 2, 3, 4, 19, 20, 32, 43, or 47, or conservatively modified variants thereof. Thus, the present invention provides full-length and subsequences of SEQ ID NO:1, 2, 3, 4, 19, 20, 32, 43, and 47 and

full-length and subsequences of conservatively modified variants of SEQ ID NO:1, 2, 3, 4, 19, 20, 32, 43, and 47. A "full-length" sequence of SEQ ID NO:1, 2, 3, 4, 19, 20, 32, 43, or 47 means the sequence of SEQ ID NO:1, 2, 3, 4, 19, 20, 32, 43 or 47, respectively. A "full-length" sequence of a conservatively modified variant of SEQ ID NO:1, 2, 3, 4, 19, 20, 32, 43 or 47 means a conservatively modified variant of SEQ ID NO:1, 2, 3, 4, 19, 20, 32, 43 or 47 respectively. The calcium-activated potassium channel proteins and peptides of the present invention can be used as immunogens for the preparation of immunodiagnostic probes for assessing increased or decreased expression of calcium-activated potassium channels in drug screening assays.

The calcium-activated potassium channel proteins of the present invention also include proteins which have substantial identity (i.e., similarity) to a calcium-activated potassium channel protein of at least N amino acids from any one of the sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO: 43, and SEQ ID NO: 47 and conservatively modified variants thereof, where N is any one of the integers selected from the group consisting of 10 to 600. Generally, the calcium-activated potassium channel proteins are at least 50, typically at least 100, preferably at least 200, more preferably at least 300, and most preferably at least 400 amino acid residues in length. Typically, the substantially similar or conservatively modified variant of the calcium-activated potassium SK or IK channel protein is a eukaryotic protein, preferably from a multicellular eukaryotes such as insects, protozoans, birds, fishes, amphibians, reptiles, or mammals.

The SK channel proteins which are substantially identical to, or a conservatively modified variant of, an SK channel protein having a sequence selected from SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:43 and SEQ ID NO:47 will specifically react, under immunologically reactive conditions, with an immunoglobulin reactive to an SK channel protein selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:43, and SEQ ID NO:47.

Similarly, IK channel proteins which are substantially identical to, or a conservatively modified variant of, an IK channel protein having a sequence selected from SEQ ID NO:32 will specifically react, under immunologically reactive conditions, with an immunoglobulin reactive to an IK channel protein such as SEQ ID NO:32. A variety of immunoassay formats may be used to assess such an immunologically specific reaction including, for example, ELISA, competitive immunoassays, radioimmunoassays, Western blots, indirect immunofluorescent assays and the like.

Alternatively, the SK channel proteins which are substantially identical to, or are a conservatively modified variant of, an SK channel protein having a sequence selected from SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:43, and SEQ ID NO:47 will comprise an amino acid sequence which has any one of the values from 60% to 100% similarity to a comparison window within the core sequence (or "core region") of an SK channel protein selected from the group consisting of SEQ ID NOS:1, 2, 3, 4, 19, 20, 43, and 47. IK channel proteins which are substantially identical to, or are a conservatively modified variant of, an IK channel protein having the sequence of SEQ ID NO:32 will comprise an amino acid sequence which has any one of the values from 60% to 100% similarity to a comparison window within the core sequence (or "core region") of the IK channel protein hIK1.

Thus, similarity is determined by reference to the core region or subsequence thereof. The core region of hSK1 (SEQ ID NO:1) is from amino acid residue 124 through 451 (SEQ ID NO:27). The core region of rSK2 (SEQ ID NO:2) is from amino acid residue 135 through 462. The core region of truncated rSK3 (SEQ ID NO:3) is from amino acid residue 109 through 436. The core region of N-terminal extended rSK3 (SEQ ID NO:43) is from 288-615. The core region of rSK1 (SEQ ID NO:4) is defined by the region which aligns with the foregoing regions. The core region of hSK2 (SEQ ID NO:19) is from amino acid residue 134 through 461. The core region of truncated hSK3 (SEQ ID NO:20) is from amino acid residue 109 through 436. The core region of N-terminal extended hSK3 (SEQ ID NO:47) is from 238-465. Thus, the core region of SEQ ID NOS:1-4, 19, 20, 43 and 47 are inclusive of and defined by the amino acid residue subsequences LSDYALIFGM (SEQ ID NO:17) at the amino proximal end

and QRKFLQAIHQ (SEQ ID NO:18) at the carboxyl proximal end. The core region of hIK1 (SEQ ID NO:32) is amino acids 25 through 351. A subsequence of the core region has a length of any one of the numbers from 10 to the length of a core sequence of SEQ ID NOS: 1, 2, 3, 4, 19, 20, 32, 43 or 47. Preferably, SK or
5 IK channel proteins comprise an amino acid sequence having at least 90% similarity over a comparison window of 20 contiguous amino acids from within the core sequence.

Similarity is also determined by reference to functional characteristics of the calcium activated channel protein. For example, the present
10 invention provides several SK3 amino acid sequences, which when expressed have virtually identical currents. cDNAs encoding rSK3 have been isolated in two different forms. The first, SEQ ID NO:44 encoding SEQ ID NO:43, is the endogenous rSK3 or N-terminal extended rSK3. The second, SEQ ID NO:16, encoding SEQ ID NO:3, is truncated relative to SEQ ID NO:43 at the N-terminus.
15 Truncated rSK3 protein (SEQ ID NO:3) also has a different C-terminus, in which the last 9 amino acids of SEQ ID NO:43 are replaced with 5 different amino acids. Although these sequences differ at both the N- and C-terminus, they express virtually identical currents. Since the N-terminal extended and truncated SK3 express the same current, the N-terminal extension not essential to channel
20 function per se but is likely involved in targeting the protein to a specific location in the cell.

Similarly, two cDNAs for hSK3 have been identified: N-terminal extended hSK3 (SEQ ID NO:48, encoding SEQ ID NO:47) and truncated hSK3 (SEQ ID NO:22, encoding SEQ ID NO:20). In addition, a similar N-terminal
25 extension may exist for SK2. Genomic sequences from the mouse for both SK2 and SK3 demonstrate that both have an extended open reading frame, which is contiguous with the amino acids sequences for which functional current expression has been demonstrated. Thus, substantially identical SK channel proteins, or conservatively modified variants thereof, are also identified on the
30 basis of functional characteristics.

The present invention provides functional SK and IK channel proteins and subsequences thereof. Functional SK channels of the present invention have a unitary conductance of between 2 and 60 pS, more usually 5

and 25 pS, and molecular weights between 40 and 100 Kd for each of the SK channel proteins which make up the SK channel, more usually 50 to 80 kD. Functional IK channels have a unitary conductance of between 20 and 80 pS, and often 30 to 60 pS. Unitary conductance may be conveniently determined using inside-out or outside-out patch clamp configurations. These configurations are particularly indicated for the study of the biophysics of ionic channels (kinetics, conductivity, selectivity, mechanism of permeation and block). Patch clamp methods are well known in the art. See, e.g., the review of Franciolini, *Patch clamp technique and biophysical study of membrane channels, Experientia*, 42(6):589-594 (1986); and Sakmann et al., *Patch clamp techniques for studying ionic channels in excitable membranes, Annual Review of Physiology*, 46:455-472 (1984).

The isolated SK and IK proteins within the scope of the present invention include those which when full-length and expressed in a cell from a quiet line, define a functionality and pharmacology indicative of an SK channel or IK channel, respectively. A quiet line is a cell line that in its native state (e.g., not expressing recombinant SK or IK channels) has low or uninteresting electric activity, e.g., a CHO cell line. For example, a control cell (without expression of a putative SK channel of the present invention) and an experimental cell (expressing a putative SK channel) are maintained under conditions standard for measurement of electrophysiological parameters as provided in the working examples disclosed herein. Each cell is treated with a calcium ionophore. Exemplary ionophores include, but are not limited to, such standard compounds as ionomycin (Sigma Chemical Co.) or A23187 (Sigma Chemical Co.). A cell is often treated with an ionophore at a concentration of about 1 μ M.

Subsequently, electrophysiological measurements of the cells are taken to detect induction of a potassium current (e.g., by radiotracer), or a change in conductance of the cell (e.g., by patch clamp), or a change in voltage (e.g., by fluorescent dye). If the presence of an ion channel is indicated by a calcium induced change, subsequent tests are used to characterize the channel as an SK channel of the present invention. Preferably, at least two characteristics are determined, more preferably at least 3, or 4 are determined. Characteristics of SK channels of the present invention are disclosed more fully herein.

For example, a cell expressing an SK channel of the present invention can have a conductance of between 2 to 30 pS, often between 2 to 25 pS, can, but not necessarily, exhibit block by apamin at a range from 10 pM to about 100 nM, can comprise an SK channel protein of about 40 to 80 kD, can exhibit sequence similarity of at least 60%, and more preferably at least 70%, 80%, 90% or 95% in an alignment with the core regions of the exemplary SK channel proteins disclosed herein, and can be specifically reactive, under immunologically reactive conditions, with an antibody raised to an exemplary SK or IK channel disclosed herein (e.g., SEQ ID NO:1-4, 19, 20, 32, 43 and 47). Such standard methods aid in the identification of SK proteins of the present invention. Cells expressing an IK channel have the same functional characteristics except they are blocked by CTX but not blocked by IBX or apamin and have a unitary conductance of between 20 and 80, often 35 to 40 pS.

Solid phase synthesis of SK or IK channel proteins of less than about 50 amino acids in length may be accomplished by attaching the C-terminal amino acid of the sequence to an insoluble support followed by sequential addition of the remaining amino acids in the sequence. Techniques for solid phase synthesis are described by Barany and Merrifield, *Solid-Phase Peptide Synthesis*; pp. 3-284 in *The Peptides: Analysis, Synthesis, Biology*. Vol. 2: *Special Methods in Peptide Synthesis, Part A.*, Merrifield, et al. *J. Am. Chem. Soc.*, 85: 2149-2156 (1963), and Stewart et al., *Solid Phase Peptide Synthesis*, 2nd ed. Pierce Chem. Co., Rockford, Ill. (1984). SK or IK channel proteins of greater length may be synthesized by condensation of the amino and carboxy termini of shorter fragments. Methods of forming peptide bonds by activation of a carboxy terminal end (e.g., by the use of the coupling reagent N,N'-dicyclohexylcarbodiimide)) is known to those of skill.

Obtaining Nucleic Acids Encoding Calcium-Activated Potassium Channel Proteins

The present invention provides isolated nucleic acids of RNA, DNA, or chimeras thereof, which encode calcium activated, SK channel proteins ("SK channel protein nucleic acids") or calcium activated, IK channel proteins ("IK channel protein nucleic acids") as discussed more fully above. Nucleic acids of the present invention can be used as probes, for example, in detecting

deficiencies in the level of mRNA, mutations in the gene (e.g., substitutions, deletions, or additions), for monitoring upregulation of SK or IK channels in drug screening assays, or for recombinant expression of SK or IK channel proteins for use as immunogens in the preparation of antibodies.

5 Nucleic acids encoding the calcium-activated potassium channel proteins of the present invention can be made using standard recombinant or synthetic techniques. With the amino acid sequences of the SK or IK channel proteins herein provided, one of skill can readily construct a variety of clones containing functionally equivalent nucleic acids, such as nucleic acids which
10 encode the same protein. Cloning methodologies to accomplish these ends, and sequencing methods to verify the sequence of nucleic acids are well known in the art. Examples of appropriate cloning and sequencing techniques, and instructions sufficient to direct persons of skill through many cloning exercises are found in Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd Ed., Vols. 1-3, Cold Spring Harbor Laboratory (1989)), *Methods in Enzymology*, Vol. 152: *Guide to Molecular Cloning Techniques* (Berger and Kimmel (eds.), San Diego: Academic Press, Inc. (1987)), or *Current Protocols in Molecular Biology*, (Ausubel, *et al.* (eds.), Greene Publishing and Wiley-Interscience, New York (1987). Product information from manufacturers of biological reagents and
15 experimental equipment also provide information useful in known biological methods. Such manufacturers include the SIGMA chemical company (Saint Louis, MO), R&D systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO
20 BRL Life Technologies, Inc. (Gaithersburg, MD), Fluka Chemica-Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), Invitrogen, San Diego, CA, and Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill.

1. Isolation of SK and IK Channel Proteins by Nucleic Acid Hybridization

30 The isolated nucleic acid compositions of this invention, whether RNA, cDNA, genomic DNA, or a hybrid of the various combinations, are isolated from biological sources or synthesized *in vitro*. Deoxynucleotides can be

prepared by any suitable method including, for example, cloning and restriction of appropriate sequences or direct chemical synthesis by methods such as the phosphotriester method of Narang *et al.* *Meth. Enzymol.* 68: 90-99 (1979); the phosphodiester method of Brown *et al.*, *Meth. Enzymol.* 68: 109-151 (1979); the diethylphosphoramidite method of Beaucage *et al.*, *Tetra. Lett.*, 22: 1859-1862 (1981); the solid phase phosphoramidite triester method described by Beaucage and Caruthers (1981), *Tetrahedron Letts.*, 22(20):1859-1862, e.g., using an automated synthesizer, e.g., as described in Needham-VanDevanter *et al.* (1984) *Nucleic Acids Res.*, 12:6159-6168; and, the solid support method of U.S. Patent No. 4,458,066. Chemical synthesis produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill would recognize that while chemical synthesis of DNA is limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

Nucleic acids encoding an SK channel protein of SEQ ID NO:1 may be obtained by amplification of a human hippocampal cDNA library using isolated nucleic acid primers having the sequence: ATGCCGGGTCCCCGGGCGGCCTGC (SEQ ID NO:5) and TCACCCGCAGTCCGAGGGGGCCAC (SEQ ID NO:6).

Nucleic acids encoding an SK channel protein of SEQ ID NO:2 may be obtained by amplification of a rat brain cDNA library using isolated nucleic acid primers having the sequence: ATGAGCAGCTGCAGGTACAACGGG (SEQ ID NO:7) and CTAGCTACTCTCAGATGAAGTTGG (SEQ ID NO:8). Nucleic acids encoding an SK channel protein of SEQ ID NO:43 may be obtained by amplification of a rat brain cDNA library using isolated nucleic acid primers having the sequence: ATGAGCTCCTGCAAATACAGCGGT (SEQ ID NO:9) and TTAGCAACTGCTTGAAGTTG (SEQ ID NO:10). Nucleic acids encoding an SK channel protein of SEQ ID NO:4 may be obtained by amplification of a rat brain cDNA library using isolated nucleic acid primers having the sequence TCAGGGAAGCCCCCGACCGTCAGT (SEQ ID NO:11) and TCACCCACAGTCTGATGCCGTGGT (SEQ ID NO:12). Nucleic acids encoding an SK channel protein of SEQ ID NO:19 may be obtained by amplification of a human hippocampal cDNA library using isolated nucleic acid primers having the

sequence: ATGAGCAGCTGCAGGTACAACG (SEQ ID NO:23) and
CTAGCTACTCTCTGATGAAGTTG (SEQ ID NO:24). Nucleic acids encoding an
SK channel protein of SEQ ID NO:20 (hSK3) may be obtained by amplification of
a human hippocampal cDNA library using isolated nucleic acid primers having the
5 sequence: ATGAGCTCCTGCAAGTATAGC (SEQ ID NO:25) and
TTAGCAACTGCTTGAAGTTGTG (SEQ ID NO:26). Nucleic acids encoding the
IK channel protein of SEQ ID NO:32 may be obtained by amplification of a human
pancreas cDNA library using isolated nucleic acid primer pairs having the
sequence: (SEQ ID NOS:38 and 39) and (SEQ ID NOS:40 and 41).

10 The isolated nucleic acids of the present invention may be cloned, or
amplified by *in vitro* methods, such as the polymerase chain reaction (PCR), the
ligase chain reaction (LCR), the transcription-based amplification system (TAS),
the self-sustained sequence replication system (SSR). A wide variety of cloning
and *in vitro* amplification methodologies are well-known to persons of skill.
15 Examples of these techniques and instructions sufficient to direct persons of skill
through many cloning exercises are found in Berger and Kimmel, *Guide to
Molecular Cloning Techniques, Methods in Enzymology* 152 Academic Press,
Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A
Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring
20 Harbor Press, NY, (Sambrook *et al.*); *Current Protocols in Molecular Biology*, F.M.
Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing
Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel);
Cashion *et al.*, U.S. patent number 5,017,478; and Carr, European Patent No.
0,246,864.

25 Examples of techniques sufficient to direct persons of skill through *in
vitro* amplification methods are found in Berger, Sambrook, and Ausubel, as well
as Mullis *et al.*, (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to
Methods and Applications* (Innis *et al.* eds) Academic Press Inc. San Diego, CA
(1990) (Innis); Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal
30 Of NIH Research* (1991) 3: 81-94; (Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA*
86: 1173; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 1874; Lomell *et al.*
(1989) *J. Clin. Chem.*, 35: 1826; Landegren *et al.*, (1988) *Science*, 241: 1077-

1080; Van Brunt (1990) *Biotechnology*, 8: 291-294; Wu and Wallace, (1989) *Gene*, 4: 560; and Barringer *et al.* (1990) *Gene*, 89:117.

Isolated nucleic acids encoding SK channel proteins comprise a nucleic acid sequence encoding an SK channel protein selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, ^{SEQ ID NO:43 and SEQ ID NO:47} and subsequences thereof. In preferred embodiments, the isolated nucleic acid encoding an SK channel protein is selected from the group consisting of: SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:21, SEQ ID NO:22, ^{SEQ ID NO:44 and SEQ ID NO:48} and subsequences thereof.

Isolated nucleic acids encoding IK channel proteins comprise a nucleic acid sequence encoding an IK channel protein such as SEQ ID NO:32, and subsequences thereof. In preferred embodiments, the isolated nucleic acid encoding an IK channel protein is SEQ ID NO:31 and subsequences thereof.

In addition to the isolated nucleic acids identified herein, the invention also includes other isolated nucleic acids encoding calcium-activated potassium channel proteins which selectively hybridize, under stringent conditions, to a nucleic acid encoding a protein selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:32, SEQ ID NO:43 and SEQ ID NO:47, and subsequences thereof. Generally, the isolated nucleic acid encoding a calcium-activated potassium channel protein of the present invention will hybridize under at least moderate stringency hybridization conditions to a nucleic acid sequence from SEQ ID NOS: 13, 14, 15, 16, 21, 22, 31, 44, or 48 which encodes the core region or subsequence thereof. Alternatively, or additionally, the isolated nucleic acid encoding the calcium-activated potassium channel protein will encode an amino acid sequence of at least 60%, 70%, 80%, or 90% similarity over the length of the core region. Conveniently, the nucleic acid encoding a subsequence of the core region is obtained from SEQ ID NOS: 13, 14, 15, 16, 21, 22, 32, 44, or 48 and is at least any one of from 15 to 400 nucleotides in length, and generally at least 250 or 300 nucleotides in length; preferably the nucleic acid will encode the entire core sequence. The nucleic acid sequence, or subsequence thereof, encoding the calcium-activated potassium channel protein comprises at least N' nucleotides in length, where N' is any one of the integers selected from the group consisting

of from 18 to 2000. Thus, the nucleic acids of the present invention comprise genomic DNA and nuclear transcripts encoding SK and IK channel proteins.

Where the nucleic acid encoding an SK or IK channel protein is to be used as nucleic acid probes, it is often desirable to label the nucleic acid with detectable labels. The labels may be incorporated by any of a number of means well known to those of skill in the art. However, in a preferred embodiment, the label is simultaneously incorporated during the amplification step in the preparation of the nucleic acids. Thus, for example, polymerase chain reaction (PCR) with labeled primers or labeled nucleotides will provide a labeled amplification product. In another preferred embodiment, transcription amplification using a labeled nucleotide (e.g., fluorescein-labeled UTP and/or CTP) incorporates a label into the transcribed nucleic acids.

Alternatively, a label may be added directly to an original nucleic acid sample (e.g., mRNA, polyA mRNA, cDNA, etc.) or to the amplification product after the amplification is completed. Means of attaching labels to nucleic acids are well known to those of skill in the art and include, for example nick translation or end-labeling (e.g., with a labeled RNA) by phosphorylation of the nucleic acid and subsequent attachment (ligation) of a nucleic acid linker joining the sample nucleic acid to a label (e.g., a fluorophore).

Detectable labels suitable for use in the present invention include any composition detectable by spectroscopic, radioisotopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include biotin for staining with labeled streptavidin conjugate, magnetic beads, fluorescent dyes (e.g., fluorescein, texas red, rhodamine, green fluorescent protein, and the like), radiolabels (e.g., ^3H , ^{125}I , ^{35}S , ^{14}C , or ^{32}P), enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

Means of detecting such labels are well known to those of skill in the art. Thus, for example, radiolabels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector

to detect emitted light. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels are detected by simply visualizing the colored label.

5 The probes are used to screen genomic or cDNA libraries from any source of interest including specific tissues (e.g., heart, brain, pancreas) and animal source such as rat, human, bird, etc. Screening techniques are known in the art and are described in the general texts cited above such as in *Sambrook and Ausubel*.

10 2. Isolation of SK and IK Channel Proteins by Immunoscreening

In addition to using nucleic acid probes for identifying novel forms of the protein claimed herein, it is possible to use antibodies to probe expression libraries. This is a well known technology. (See Young and Davis, 1982 Efficient isolation of genes using antibody probes *Proc. Natl. Acad. Sci., U.S.A.* 80:1194-1198.) In general, a cDNA expression library maybe prepared from commercially available kits or using readily available components. Phage vectors are preferred, but a variety of other vectors are available for the expression of protein. such vectors include but are not limited to yeast, animal cells and *Xenopus* oocytes. One selects mRNA from a source that is enriched with the target protein and creates cDNA which is then ligated into a vector and transformed into the library host cells for immunoscreening. Screening involves binding and visualization of antibodies bound to specific proteins on cells or immobilized on a solid support such as nitrocellulose or nylon membranes. Positive clones are selected for purification to homogeneity and the isolated cDNA then prepared for expression in the desired host cells. A good general review of this technology can be found in *Methods of Cell Biology Vol 37* entitled *Antibodies in Cell Biology*, Ed. DJ Asai pp 369-382, 1993.

25 When choosing to obtain calcium activated channel proteins antibodies selective for the entire protein or portions can be used. Suitable peptide sequences include, but are not limited to, GHRRALFEKRKRLSDY (SEQ ID NO:28), FTDASSRSIGAL (SEQ ID NO:29), and

ARKLELTKAEKHVHNFMMDTQLTKR (SEQ ID NO:30) or
ARKLELTKAEKHVHNFMMDTQLTK (SEQ ID NO:42).

Nucleic Acid Assays

5 This invention also provides methods of detecting and/or quantifying SK or IK channel protein expression by assaying for the gene transcript (e.g., nuclear RNA, mRNA). The assay can be for the presence or absence of the normal gene or gene product, for the presence or absence of an abnormal gene or gene product, or quantification of the transcription levels of normal or abnormal SK or IK channel protein gene product.

10 In a preferred embodiment, nucleic acid assays are performed with a sample of nucleic acid isolated from the organism to be tested. In the simplest embodiment, such a nucleic acid sample is the total mRNA isolated from a biological sample. The nucleic acid (e.g., either genomic DNA or mRNA) may be isolated from the sample according to any of a number of methods well known to those of skill in the art.

15 Methods of isolating total DNA or mRNA for use in, *inter alia*, a nucleic acid assay are well known to those of skill in the art. For example, methods of isolation and purification of nucleic acids are described in detail in Chapter 3 of *Laboratory Techniques in Biochemistry and Molecular Biology: Hybridization With Nucleic Acid Probes, Part I. Theory and Nucleic Acid Preparation*, P. Tijssen, ed. Elsevier, N.Y. (1993). One of skill will appreciate that where alterations in the copy number of the gene encoding an SK or IK channel protein is to be detected genomic DNA is preferably isolated. Conversely, where expression levels of a gene or genes are to be detected, preferably RNA (mRNA) is isolated.

25 Frequently, it is desirable to amplify the nucleic acid sample prior to hybridization. One of skill in the art will appreciate that whatever amplification method is used, if a quantitative result is desired, care must be taken to use a method that maintains or controls for the relative frequencies of the amplified nucleic acids. Methods of "quantitative" amplification are well known to those of skill in the art. For example, quantitative PCR involves simultaneously co-amplifying a known quantity of a control sequence using the same primers. This

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provides an internal standard that may be used to calibrate the PCR reaction. The high density array may then include probes specific to the internal standard for quantification of the amplified nucleic acid. Detailed protocols for quantitative PCR are provided in *PCR Protocols, A Guide to Methods and Applications*, Innis et al., Academic Press, Inc. N.Y., (1990).

5 The method of detecting the presence of a nucleic acid sequence encoding an SK channel protein generally comprises: (a) contacting the biological sample, under stringent hybridization conditions, with a nucleic acid probe comprising a nucleic acid segment which selectively hybridizes to a nucleic acid
10 sequence (target) encoding an SK channel protein selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:43, and SEQ ID NO:47; (b) allowing the probe to specifically hybridize to the nucleic acid encoding an SK channel protein to form a hybridization complex, wherein detection of the hybridization complex is an
15 indication of the presence of the SK nucleic acid sequence in the sample. Detection of an IK channel protein is accomplished in a similar fashion using a nucleic acid segment which selectively hybridizes to a nucleic acid sequence encoding an IK channel protein of SEQ ID NO:32.

20 The nucleic acid segment of the probe is a subsequence of at least N" contiguous nucleotides in length from a nucleic acid encoding an SK channel selected from the group consisting of SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:44, and SEQ ID NO:48, and complementary sequences thereof. N" is an any one of the
25 integers selected from the group consisting of each of the integers from 15 to 1500. For detecting the presence of an IK channel protein the nucleic acid segment is a subsequence of at least N" contiguous nucleotides in length from a nucleic acid encoding an IK channel of SEQ ID NO:31. "Contiguous nucleotides" from a referenced nucleic acid means a sequence of nucleotides having the same
30 order and directly adjacent to the same nucleotides (i.e., without additions or deletions) as in the referenced nucleic acid. Typically, the nucleic acid segment is at least 18 nucleotides in length. The preferred length of the nucleic acid probe is from 24 to 200 nucleotides in length.

In particularly preferred embodiments, the nucleic acid segment is derived from a nucleic acid which encodes a core region from a protein selected from the group consisting of SEQ ID NO:1, 2, 3, 4, 19, 20, 32, 43 and 47. Conveniently, the nucleic acid which encodes the core region is a subsequence of a nucleic acid selected from the group consisting of: SEQ ID NOS: 13, 14, 15, 16, 21, 22, 31, 44, 48, and complementary sequences thereof. Usually, and particularly for cross-species hybridization, the nucleic acid segment would encode an amino acid sequence from within the core region and will be at least 250 nucleotides in length, most preferably will encode the entirety of the core region, and/or will hybridize to the target sequence under moderate stringency hybridization conditions.

Those of skill will appreciate that nucleic acid sequences of the probe will be chosen so as not to interfere in the selective hybridization of the nucleic acid segment to the target. Thus, for example, any additional nucleotides attached to the nucleic acid segment will generally be chosen so as not to selectively hybridize, under stringent conditions, to the nucleic acid target (potential false negative), nor to nucleic acids not encoding an SK or IK channel proteins or peptides (potential false positive). The use of negative and positive controls to ensure selectivity and specificity is known to those of skill. In general, the length of the probe should be kept to the minimum length necessary to achieve the desired results. The length of the nucleic acid encoding an SK or IK channel protein or peptide (i.e., the "SK channel protein nucleic acid" or "IK channel protein nucleic acid", respectively) is discussed more fully, *supra*, but is preferably at least 30 nucleotides in length.

A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in Berger and Kimmel, (1987), *supra*.; "Nucleic Acid Hybridization, A Practical Approach" (Hames, B.D. and Higgins, S.J. (eds.), IRL Press, 1985; Gall and Pardue, (*Proc. Natl. Acad. Sci., U.S.A.* 63:378-383 (1969)); and John, Burnsteil and Jones (*Nature*, 223:582-587 (1969)).

Sandwich assays are commercially useful hybridization assays for detecting or isolating nucleic acid sequences. Such assays utilize a "capture"

nucleic acid covalently immobilized to a solid support and a labelled "signal" nucleic acid in solution. The biological sample will provide the target nucleic acid. The "capture" nucleic acid probe and the "signal" nucleic acid probe hybridize with the target nucleic acid to form a "sandwich" hybridization complex. To be effective, the signal nucleic acid cannot hybridize with the capture nucleic acid.

In *in situ* hybridization, the target nucleic acid is liberated from its cellular surroundings in such as to be available for hybridization within the cell while preserving the cellular morphology for subsequent interpretation and analysis. The following articles provide an overview of the art of *in situ* hybridization: Singer *et al.*, *Biotechniques* 4(3):230-250 (1986); Haase *et al.*, *Methods in Virology*, Vol. VII, pp. 189-226 (1984); Wilkinson, "The theory and practice of *in situ* hybridization" In: *In situ Hybridization*, Ed. D.G. Wilkinson. IRL Press, Oxford University Press, Oxford; and *Nucleic Acid Hybridization: A Practical Approach*, Ed. Hames, B.D. and Higgins, S.J., IRL Press (1987).

Typically, labelled signal nucleic acids are used to detect hybridization. Complementary nucleic acids or signal nucleic acids may be labelled by any one of several methods typically used to detect the presence of hybridized oligonucleotides. The most common method of detection is the use of autoradiography with ^3H , ^{125}I , ^{35}S , ^{14}C , or ^{32}P -labelled probes or the like. Other labels include ligands which bind to labelled antibodies, fluorophores, chemiluminescent agents, enzymes, and antibodies which can serve as specific binding pair members for a labelled ligand.

The label may also allow for the indirect detection of the hybridization complex. For example, where the label is a hapten or antigen, the sample can be detected by using antibodies. In these systems, a signal is generated by attaching fluorescent or enzyme molecules to the antibodies or, in some cases, by attachment to a radioactive label. (Tijssen, "Practice and Theory of Enzyme Immunoassays," *Laboratory Techniques in Biochemistry and Molecular Biology* (Burdon, van Knippenberg (eds.), Elsevier, pp. 9-20 (1985)).

The detectable label used in nucleic acids of the present invention may be incorporated by any of a number of means known to those of skill in the art, e.g., as discussed *supra*. Means of detecting such labels are well known to those of skill in the art.

The sensitivity of the hybridization assays may be enhanced through the use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. Examples of such systems include the polymerase chain reaction (PCR) system and the ligase chain reaction (LCR) system. Other methods known in the art are the nucleic acid sequence based amplification (NASBA, Cangene, Mississauga, Ontario) and Q-Beta Replicase systems.

Those of skill will appreciate that abnormal expression levels or abnormal expression products (e.g., mutated transcripts, truncated or non-sense proteins) are identified by comparison to normal expression levels and normal expression products. Normal levels of expression or normal expression products can be determined for any particular population, subpopulation, or group of organisms according to standard methods known to those of skill in the art. Typically this involves identifying healthy organisms (i.e., organisms with a functional SK or IK channel protein as indicated by such properties as conductance and calcium sensitivity) and measuring expression levels of the SK or IK channel protein gene (as described herein) or sequencing the gene, mRNA, or reverse transcribed cDNA, to obtain typical (normal) sequence variations. Application of standard statistical methods used in molecular genetics permits determination of baseline levels of expression, and normal gene products as well as significant deviations from such baseline levels.

Nucleic Acid Assay Kits

The nucleic acids of this invention can be included in a kit which can be used to determine in a biological sample the presence or absence of the normal gene or gene product encoding an SK or IK channel of the present invention, for the presence or absence of an abnormal gene or gene product encoding an SK or IK channel, or quantification of the transcription levels of normal or abnormal SK or IK channel protein gene product. The kit typically includes a stable preparation of nucleic acid probes for performing the assay of the present invention. Further, the kit may also include a hybridization solution in either dry or liquid form for the hybridization of probes to target calcium-activated potassium channel proteins or calcium-activated potassium channel protein nucleic acids of the present invention, a solution for washing and removing

undesirable and non-hybridized nucleic acids, a substrate for detecting the hybridization complex, and/or instructions for performing and interpreting the assay.

Expression of Nucleic Acids

5 Once the nucleic acids encoding an SK or IK channel protein of the present invention are isolated and cloned, one may express the desired protein in a recombinantly engineered cell such as bacteria, yeast, insect (especially employing baculoviral vectors), and mammalian cells. A "recombinant protein" is a protein produced using cells that do not have in their native form an
10 endogenous copy of the DNA able to express the protein. The cells produce the recombinant protein because they have been genetically altered by the introduction of the appropriate isolated nucleic acid sequence (e.g., a vector comprising an SK or IK channel protein nucleic acid).

15 It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of DNA encoding SK or IK channel proteins. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made.

20 In brief summary, the expression of natural or synthetic nucleic acids encoding calcium-activated potassium channel proteins of the present invention will typically be achieved by operably linking the DNA or cDNA to a promoter (which is either constitutive or inducible), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for
25 regulation of the expression of the DNA encoding the SK or IK channel protein. To obtain high level expression of a cloned gene, it is desirable to construct expression vectors which contain, at the minimum, a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. One of skill would recognize that
30 modifications can be made to an SK or IK channel protein without diminishing its biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a fusion protein. Such

modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids (e.g., poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification sequences.

5 1. Expression in Prokaryotes

Examples of regulatory regions suitable for this purpose in *E. coli* are the promoter and operator region of the *E. coli* tryptophan biosynthetic pathway as described by Yanofsky, *Bacteriol.* 158:1018-1024 (1984), and the leftward promoter of phage lambda (P_L) as described by Herskowitz and Hagen, 10 *Ann. Rev. Genet.*, 14:399-445 (1980). The inclusion of selection markers in DNA vectors transfected in *E. coli* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol. See, Sambrook, *et al.* for details concerning selection markers for use in *E. coli*.

15 The vector is selected to allow introduction into the appropriate host cell. Bacterial vectors are typically of plasmid or phage origin. Appropriate bacterial cells are infected with phage vector particles or transfected with naked phage vector DNA. If a plasmid vector is used, the bacterial cells are transfected with the plasmid vector DNA. Expression systems for expressing SK channel proteins are available using *E. coli*, *Bacillus sp.* and *Salmonella* (Palva, *et al.*, 20 *Gene* 22:229-235 (1983); Mosbach, *et al.*, *Nature* 302:543-545 (1983)).

When expressing SK or IK channel proteins in *S. typhimurium*, one should be aware of the inherent instability of plasmid vectors. To circumvent this, the foreign gene can be incorporated into a nonessential region of the host chromosome. This is achieved by first inserting the gene into a plasmid such that 25 it is flanked by regions of DNA homologous to the insertion site in the *Salmonella* chromosome. After introduction of the plasmid into the *S. typhimurium*, the foreign gene is incorporated into the chromosome by homologous recombination between the flanking sequences and chromosomal DNA.

30 An example of how this can be achieved is based on the *his* operon of *Salmonella*. Two steps are involved in this process. First, a segment of the *his* operon must be deleted in the *Salmonella* strain selected as the carrier. Second, a plasmid carrying the deleted *his* region downstream of the gene

encoding the SK or IK channel protein is transfected into the *his Salmonella* strain. Integration of both the *his* sequences and a gene encoding an SK or IK channel protein occurs, resulting in recombinant strains which can be selected as *his*⁺.

5 Detection of the expressed protein is achieved by methods known in the art and include, for example, radioimmunoassays, Western blotting techniques or immunoprecipitation. Purification from *E. coli* can be achieved following procedures described in U.S. Patent No. 4,511,503.

2. Expression in Eukaryotes

10 A variety of eukaryotic expression systems such as yeast, insect cell lines, bird, fish, frog, and mammalian cells, are known to those of skill in the art. As explained briefly below, SK or IK channel proteins of the present invention may be expressed in these eukaryotic systems. Expression of SK or IK channels in eukaryotes is particularly preferred.

15 Synthesis of heterologous proteins in yeast is well known. *Methods in Yeast Genetics*, Sherman, F., *et al.*, Cold Spring Harbor Laboratory, (1982) is a well recognized work describing the various methods available to produce the protein in yeast. Suitable vectors usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as
20 desired. For instance, suitable vectors are described in the literature (Botstein, *et al.*, 1979, *Gene*, 8:17-24; Broach, *et al.*, 1979, *Gene*, 8:121-133).

25 Two procedures are used in transfecting yeast cells. In one case, yeast cells are first converted into protoplasts using zymolyase, lyticase or glucanase, followed by addition of DNA and polyethylene glycol (PEG). The PEG-treated protoplasts are then regenerated in a 3% agar medium under selective conditions. Details of this procedure are given in the papers by J.D. Beggs, 1978, *Nature* (London), 275:104-109; and Hinnen, A., *et al.*, 1978, *Proc. Natl. Acad. Sci. USA*, 75:1929-1933. The second procedure does not involve
30 removal of the cell wall. Instead the cells are treated with lithium chloride or acetate and PEG and put on selective plates (Ito, H., *et al.*, 1983, *J. Bact.*, 153:163-168).

The calcium-activated potassium channel proteins of the present invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysates. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay of other standard immunoassay techniques.

The sequences encoding the calcium-activated potassium channel proteins can also be ligated to various expression vectors for use in transfecting cell cultures of, for instance, mammalian, insect, bird, amphibian, or fish origin. Illustrative of cell cultures useful for the production of the peptides are mammalian cells. Mammalian cell systems often will be in the form of monolayers of cells although mammalian cell suspensions may also be used. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the HEK293, BHK21, and CHO cell lines, and various human cells such as COS cell lines, HeLa cells, myeloma cell lines, Jurkat cells. In some embodiments, *Xenopus* oocytes are used. Those of skill will recognize that preferred cell lines for expressing SK or IK channels substantially lack conductances which compete with those provided by the calcium-activated potassium channels of the present invention (i.e., "quiet lines"). Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter (e.g., the CMV promoter, a HSV *tk* promoter or *pgk* (phosphoglycerate kinase) promoter), an enhancer (Queen *et al.* (1986) *Immunol. Rev.* 89:49), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (e.g., an SV40 large T Ag poly A addition site), and transcriptional terminator sequences. Other animal cells useful for production of SK channel proteins are available, for instance, from the American Type Culture Collection Catalogue of Cell Lines and Hybridomas (7th edition, 1992).

Appropriate vectors for expressing SK or IK channel proteins in insect cells are usually derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae, silkworm, armyworm, moth and *Drosophila* cell lines such as a Schneider cell line (See Schneider *J. Embryol. Exp. Morphol.* 27:353-365 (1987)).

As indicated above, the vector, e.g., a plasmid, which is used to transfect the host cell, preferably contains DNA sequences to initiate transcription and sequences to control the translation of the protein. These sequences are referred to as expression control sequences.

5 As with yeast, when higher animal host cells are employed, polyadenylation or transcription terminator sequences from known mammalian genes need to be incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An
10 example of a splicing sequence is the VP1 intron from SV40 (Sprague, J. *et al.*, 1983, *J. Virol.* 45: 773-781).

Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors. Saveria-Campo, M., 1985, "Bovine Papilloma virus DNA a
15 Eukaryotic Cloning Vector" in *DNA Cloning Vol. II a Practical Approach* Ed. D.M. Glover, IRL Press, Arlington, Virginia pp. 213-238.

The host cells are competent or rendered competent for transfection by various means. There are several well-known methods of introducing DNA into animal cells. These include: calcium phosphate precipitation, fusion of the
20 recipient cells with bacterial protoplasts containing the DNA, treatment of the recipient cells with liposomes containing the DNA, DEAE dextran, electroporation and micro-injection of the DNA directly into the cells. The transfected cells are cultured by means well known in the art. *Biochemical Methods in Cell Culture and Virology*, Kuchler, R.J., Dowden, Hutchinson and Ross, Inc., (1977). The
25 expressed proteins are recovered by well known mechanical, chemical or enzymatic means.

Purification of Expressed Peptides

The SK or IK channel proteins of the present invention which are produced by recombinant DNA technology may be purified by standard
30 techniques well known to those of skill in the art. Recombinantly produced SK or IK channel proteins can be directly expressed or expressed as a fusion protein. The recombinant calcium-activated potassium channel protein of the present

invention is purified by a combination of cell lysis (e.g., sonication) and affinity chromatography. For fusion products, subsequent digestion of the fusion protein with an appropriate proteolytic enzyme releases the desired recombinant calcium-activated potassium channel protein.

5 The calcium-activated potassium channel proteins of this invention, recombinant or synthetic, may be purified to substantial purity by standard techniques well known in the art, including selective precipitation with such substances as ammonium sulfate, column chromatography, immunopurification methods, and others. See, for instance, R. Scopes, *Protein Purification:*
10 *Principles and Practice*, Springer-Verlag: New York (1982); Deutscher, *Guide to Protein Purification*, Academic Press, 1990. For example, the proteins of this invention may be purified by immunoaffinity columns using antibodies raised to the SK or IK channel proteins as described herein.

Antibodies to Calcium-Activated Potassium Channel Proteins

15 Antibodies are raised to the SK or IK channel protein of the present invention, including individual, allelic, strain, or species variants, and fragments thereof, both in their naturally occurring (full-length) forms and in recombinant forms. Additionally, antibodies are raised to these proteins in either their native configurations or in non-native configurations. Anti-idiotypic antibodies can also
20 be generated. Many methods of making antibodies are known to persons of skill. The following discussion is presented as a general overview of the techniques available; however, one of skill will recognize that many variations upon the following methods are known.

A. Antibody Production

25 A number of immunogens are used to produce antibodies specifically reactive with an SK or IK channel protein. An isolated recombinant, synthetic, or native SK or IK channel protein of 5 amino acids in length or greater, and selected from a subsequence of SEQ ID NO:1, 2, 3, 4, 19, 20, 32, 43, or 47 are the preferred immunogens (antigen) for the production of monoclonal or
30 polyclonal antibodies. Those of skill will readily understand that the calcium-activated potassium channel proteins of the present invention are typically

denatured prior to formation of antibodies for screening expression libraries or other assays in which a putative calcium-activated potassium channel protein of the present invention is expressed or denatured in a non-native secondary, tertiary, or quaternary structure. Exemplary proteins for use as immunogens include, but are not limited to, GHRRALFEKRKRLSDY (SEQ ID NO:28), FTDASSRSIGAL (SEQ ID NO:29), ARKLELTKAEKHVHNFMMDTQLTKR (SEQ ID NO:30), and ARKLELTKAEKHVHNFMMDTQLTK (SEQ ID NO:42). In one class of preferred embodiments, an immunogenic protein conjugate is also included as an immunogen. Naturally occurring SK or IK channel proteins are also used either in pure or impure form.

The SK or IK channel protein is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies can be generated for subsequent use in immunoassays to measure the presence and quantity of the calcium-activated potassium channel protein. Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen (antigen), preferably a purified SK or IK channel protein, an SK or IK channel protein coupled to an appropriate carrier (e.g., GST, keyhole limpet hemanocyanin, etc.), or an SK or IK channel protein incorporated into an immunization vector such as a recombinant vaccinia virus (see, U.S. Patent No. 4,722,848) is mixed with an adjuvant and animals are immunized with the mixture. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the calcium-activated potassium channel protein of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the SK or IK channel protein is performed where desired (see, e.g., Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY).

Antibodies, including binding fragments and single chain recombinant versions thereof, against predetermined fragments of SK or IK channel protein are raised by immunizing animals, e.g., with conjugates of the fragments with carrier proteins as described above. Typically, the immunogen of

interest is an SK or IK channel protein of at least about 5 amino acids, more typically the SK or IK channel protein is 10 amino acids in length, preferably, 15 amino acids in length and more preferably the calcium-activated potassium channel protein is 20 amino acids in length or greater. The peptides are typically coupled to a carrier protein (e.g., as a fusion protein), or are recombinantly expressed in an immunization vector. Antigenic determinants on peptides to which antibodies bind are typically 3 to 10 amino acids in length.

Monoclonal antibodies are prepared from cells secreting the desired antibody. Monoclonal antibodies are screened for binding to an SK or IK channel protein from which the immunogen was derived. Specific monoclonal and polyclonal antibodies will usually bind with a K_D of at least about 0.1 mM, more usually at least about 50 μ M, and most preferably at least about 1 μ M or better.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, etc. Description of techniques for preparing such monoclonal antibodies are found in, e.g., Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane, *Supra*; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256: 495-497. Summarized briefly, this method proceeds by injecting an animal with an immunogen comprising an SK or IK channel protein. The animal is then sacrificed and cells taken from its spleen, which are fused with myeloma cells. The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*. The population of hybridomas is then screened to isolate individual clones, each of which secrete a single antibody species to the immunogen. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

Alternative methods of immortalization include transfection with Epstein Barr Virus, oncogenes, or retroviruses, or other methods known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells is enhanced by various techniques.

including injection into the peritoneal cavity of a vertebrate (preferably mammalian) host. The SK or IK channel proteins and antibodies of the present invention are used with or without modification, and include chimeric antibodies, such as humanized murine antibodies.

5 Other suitable techniques involve selection of libraries of recombinant antibodies in phage or similar vectors (*see, e.g., Huse et al. (1989) Science 246: 1275-1281; and Ward, et al. (1989) Nature 341: 544-546; and Vaughan et al. (1996) Nature Biotechnology, 14: 309-314*). Alternatively, high avidity human monoclonal antibodies can be obtained from transgenic mice comprising fragments of the unrearranged human heavy and light chain Ig loci (i.e., minilocus transgenic mice). Fishwild *et al., Nature Biotech., 14:845-851* (1996).

15 Frequently, the SK or IK channel proteins and antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Patent Nos. 20 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced. See, Cabilly, U.S. Patent No. 4,816,567; and Queen *et al. (1989) Proc. Nat'l Acad. Sci. USA 86: 10029-10033*.

25 The antibodies of this invention are also used for affinity chromatography in isolating SK or IK channel proteins. Columns are prepared, *e.g., with the antibodies linked to a solid support, e.g., particles, such as agarose, Sephadex, or the like, where a cell lysate is passed through the column, washed, and treated with increasing concentrations of a mild denaturant, whereby purified SK or IK channel protein are released.*

30 The antibodies can be used to screen expression libraries for particular expression products such as normal or abnormal human SK or IK channel protein. Usually the antibodies in such a procedure are labeled with a moiety allowing easy detection of presence of antigen by antibody binding.

Antibodies raised against SK or IK channel protein can also be used to raise anti-idiotypic antibodies. These are useful for detecting or diagnosing various pathological conditions related to the presence of the respective antigens.

B. Human or Humanized (Chimeric) Antibody Production

5 The anti-SK or anti-IK channel protein antibodies of this invention can also be administered to a mammal (e.g., a human patient) for therapeutic purposes (e.g., as targeting molecules when conjugated or fused to effector molecules such as labels, cytotoxins, enzymes, growth factors, drugs, etc.). Antibodies administered to an organism other than the species in which they are
10 raised are often immunogenic. Thus, for example, murine antibodies administered to a human often induce an immunologic response against the antibody (e.g., the human anti-mouse antibody (HAMA) response) on multiple administrations. The immunogenic properties of the antibody are reduced by altering portions, or all, of the antibody into characteristically human sequences
15 thereby producing chimeric or human antibodies, respectively.

i) Humanized (Chimeric) Antibodies

Humanized (chimeric) antibodies are immunoglobulin molecules comprising a human and non-human portion. More specifically, the antigen combining region (or variable region) of a humanized chimeric antibody is derived
20 from a non-human source (e.g., murine) and the constant region of the chimeric antibody (which confers biological effector function to the immunoglobulin) is derived from a human source. The humanized chimeric antibody should have the antigen binding specificity of the non-human antibody molecule and the effector function conferred by the human antibody molecule. A large number of methods
25 of generating chimeric antibodies are well known to those of skill in the art (see, e.g., U.S. Patent Nos: 5,502,167, 5,500,362, 5,491,088, 5,482,856, 5,472,693, 5,354,847, 5,292,867, 5,231,026, 5,204,244, 5,202,238, 5,169,939, 5,081,235, 5,075,431, and 4,975,369). Detailed methods for preparation of chimeric (humanized) antibodies can be found in U.S. Patent 5,482,856.

ii) Human Antibodies

In another embodiment, this invention provides for fully human anti-SK channel protein antibodies. Human antibodies consist entirely of characteristically human polypeptide sequences. The human anti-SK or anti-IK channel protein antibodies of this invention can be produced in using a wide variety of methods (see, e.g., Larrick *et al.*, U.S. Pat. No. 5,001,065, for review).

In preferred embodiments, the human anti-SK channel protein antibodies of the present invention are usually produced initially in trioma cells. Genes encoding the antibodies are then cloned and expressed in other cells, particularly, nonhuman mammalian cells. The general approach for producing human antibodies by trioma technology has been described by Ostberg *et al.* (1983), *Hybridoma* 2: 361-367, Ostberg, U.S. Pat. No. 4,634,664, and Engelman *et al.*, U.S. Pat. No. 4,634,666. The antibody-producing cell lines obtained by this method are called triomas because they are descended from three cells; two human and one mouse. Triomas have been found to produce antibody more stably than ordinary hybridomas made from human cells.

The genes encoding the heavy and light chains of immunoglobulins secreted by trioma cell lines are cloned according to methods, including the polymerase chain reaction, known in the art (see, e.g., Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, N.Y., 1989; Berger & Kimmel, *Methods in Enzymology*, Vol. 152: *Guide to Molecular Cloning Techniques*, Academic Press, Inc., San Diego, Calif., 1987; Co *et al.* (1992) *J. Immunol.*, 148: 1149). For example, genes encoding heavy and light chains are cloned from a trioma's genomic DNA or cDNA produced by reverse transcription of the trioma's RNA. Cloning is accomplished by conventional techniques including the use of PCR primers that hybridize to the sequences flanking or overlapping the genes, or segments of genes, to be cloned.

Calcium-Activated Potassium Channel Protein Immunoassays

Immunoassays for SK and IK channel proteins can be used for at least two different purposes. They can be used to determine the relatedness of the protein by virtue of their being able to cross-react immunologically or for detection of the presence or absence of the channel proteins.

When determining if an unknown protein is related to the channel proteins of this invention, a variety of assays can be used. For example and preferred is a competitive immunoassay to test for cross-reactivity.

For example, the protein of SEQ ID NO:2 or 32 can be immobilized to a solid support. Proteins or peptides are added to the assay which compete with the binding of the antisera to the immobilized antigen. The ability of the above proteins to compete with the binding of the antisera to the immobilized protein is compared to the protein thought to be related to the test protein.

To assure that the antisera being tested is specific or selectively binding to a particular protein, it will be tested for cross-reactivity to other closely related proteins. This allows for the production of sera that will distinguish between small, intermediate and large conductance channels. The percent crossreactivity for the above proteins can be calculated, using standard calculations. Those antisera with less than 10% crossreactivity with each of the proteins listed above are selected and pooled. The cross-reacting antibodies are optionally removed from the pooled antisera by immunoabsorption with the above-listed proteins.

The immunoabsorbed and pooled antisera are then used in a competitive binding immunoassay as described above to compare a second protein to the claimed or prototype immunogen protein. In order to make this comparison, the two proteins are each assayed at a wide range of concentrations and the amount of each protein required to inhibit 50% of the binding of the antisera to the immobilized protein is determined. If the amount of protein required is less than twice the amount of the prototype protein, then the second protein is said to specifically bind to an antibody generated to the prototype immunogen. Where the antibodies are generated to a short peptide, the test proteins are optionally denatured to fully test for selective binding. In situations where the target peptide is not readily accessible to the antibodies because the target peptide is part of a larger protein, it is proper to measure the relatedness of test proteins against prototype proteins of similar size, e.g., one would test a full length monomer against a prototype, full length monomer even though the antisera was generated against a peptide of the prototype monomer. This simplifies the reading of the test results and avoids having to take into account

conformational problems and molecular weight/molar concentrations in the determination of the data generated from the competitive immunoassays.

Means of detecting the SK or IK channel proteins of the present invention are not critical aspects of the present invention. In a preferred embodiment, the SK or IK channel proteins are detected and/or quantified using any of a number of well recognized immunological binding assays (see, e.g., U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also *Methods in Cell Biology Volume 37: Antibodies in Cell Biology*, Asai, ed. Academic Press, Inc. New York (1993); *Basic and Clinical Immunology* 7th Edition, Stites & Terr, eds. (1991). Immunological binding assays (or immunoassays) typically utilize a "capture agent" to specifically bind to and often immobilize the analyte (in this case a calcium-activated potassium channel protein). The capture agent is a moiety that specifically binds to the analyte. In a preferred embodiment, the capture agent is an antibody that specifically binds a calcium-activated potassium channel protein(s) of the present invention. The antibody (anti-SK or anti-IK channel protein antibody) may be produced by any of a number of means known to those of skill in the art as described herein.

Immunoassays also often utilize a labeling agent to specifically bind to and label the binding complex formed by the capture agent and the analyte. The labeling agent may itself be one of the moieties comprising the antibody/analyte complex. Thus, the labeling agent may be a labeled SK or IK channel protein or a labeled anti-SK or anti-IK channel protein antibody. Alternatively, the labeling agent may be a third moiety, such as another antibody, that specifically binds to the antibody/SK or antibody/IK channel protein complex.

In a preferred embodiment, the labeling agent is a second SK or IK channel protein antibody bearing a label. Alternatively, the second SK or IK channel protein antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second can be modified with a detectable moiety, such as biotin, to which a third labeled molecule can specifically bind, such as enzyme-labeled streptavidin.

Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the label agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species (see, generally Kronval, *et al.* (1973) *J. Immunol.*, 111: 1401-1406, and Akerstrom, *et al.* (1985) *J. Immunol.*, 135: 2589-2542).

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, analyte, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

While the details of the immunoassays of the present invention may vary with the particular format employed, the method of detecting an SK or IK channel protein in a biological sample generally comprises the steps of contacting the biological sample with an antibody which specifically reacts, under immunologically reactive conditions, to the SK or IK channel protein. The antibody is allowed to bind to the SK or IK channel protein under immunologically reactive conditions, and the presence of the bound antibody is detected directly or indirectly.

A. Non-Competitive Assay Formats

Immunoassays for detecting SK or IK channel proteins of the present invention include competitive and noncompetitive formats. Noncompetitive immunoassays are assays in which the amount of captured analyte (in this case an SK or IK channel protein) is directly measured. In one preferred "sandwich" assay, for example, the capture agent (anti-SK or anti-IK channel protein antibodies) can be bound directly to a solid substrate where they are immobilized. These immobilized antibodies then capture SK or IK channel protein present in the test sample. The SK or IK channel protein thus immobilized is then bound by a labeling agent, such as a second human SK or IK channel protein antibody

bearing a label. Alternatively, the second SK or IK channel protein antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second can be modified with a detectable moiety, such as biotin, to which a third labeled molecule can specifically bind, such as enzyme-labeled streptavidin.

B. Competitive Assay Formats

In competitive assays, the amount of analyte (SK or IK channel protein) present in the sample is measured indirectly by measuring the amount of an added (exogenous) analyte (SK or IK channel protein) displaced (or competed away) from a capture agent (anti-SK or anti-IK channel protein antibody) by the analyte present in the sample. In one competitive assay, a known amount of, in this case, SK or IK channel protein is added to the sample and the sample is then contacted with a capture agent, in this case an antibody that specifically binds the SK or IK channel protein. The amount of SK or IK channel protein bound to the antibody is inversely proportional to the concentration of SK or IK channel protein present in the sample.

In a particularly preferred embodiment, the antibody is immobilized on a solid substrate. The amount of SK or IK channel protein bound to the antibody may be determined either by measuring the amount of SK or IK channel protein present in the corresponding SK or IK channel protein/antibody complex, or alternatively by measuring the amount of remaining uncomplexed SK or IK channel protein. The amount of SK or IK channel protein may be detected by providing a labeled SK or IK channel protein molecule.

A hapten inhibition assay is another preferred competitive assay. In this assay a known analyte, in this case the SK or IK channel protein is immobilized on a solid substrate. A known amount of anti-SK or anti-IK channel protein antibody, respectively, is added to the sample, and the sample is then contacted with the immobilized SK or IK channel protein. In this case, the amount of anti-SK or anti-IK channel protein antibody bound to the immobilized SK or IK channel protein is inversely proportional to the amount of SK or IK channel protein present in the sample. Again the amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of

the antibody that remains in solution. Detection may be direct where the antibody is labeled or indirect by the subsequent addition of a labeled moiety that specifically binds to the antibody as described above.

C. Other Assay Formats

5 In a particularly preferred embodiment, Western blot (immunoblot) analysis is used to detect and quantify the presence of an SK or IK channel protein in the sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a
10 nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind SK channel protein. The anti-SK or anti-IK channel protein antibodies specifically bind to the SK or IK channel proteins, respectively, on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (e.g., labeled
15 sheep anti-mouse antibodies) that specifically bind to the anti-SK or anti-IK channel protein.

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (e.g., antibodies) and release encapsulated reagents or markers. The released chemicals are then detected
20 according to standard techniques (see, Monroe *et al.* (1986) *Amer. Clin. Prod. Rev.* 5:34-41).

D. Labels

The particular label or detectable group used in the assay is not a critical aspect of the invention, so long as it does not significantly interfere with
25 the specific binding of the antibody used in the assay. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic,
30 photochemical, biochemical, immunochemical, radioisotopic, electrical, optical or

chemical means. Useful labels in the present invention include those used in labeling of nucleic acids as discussed, *supra*.

5 The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (e.g., biotin) is covalently bound to the molecule.
10 The ligand then binds to an anti-ligand (e.g., streptavidin) molecule which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti-ligands can be used. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the
15 labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

The molecules can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases,
20 esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol. For a review of various labeling or signal producing systems which may be used, see, U.S. Patent No.
25 4,391,904).

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome
30 with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by

providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen-coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

Immunoassay Detection Kits

The present invention also provides for kits for the diagnosis of organisms (e.g., patients) with a deficiency in the levels of expressed SK or IK channel protein. The kits preferably include one or more reagents for detecting an the amount of SK or IK channel protein in a mammal. Preferred reagents include antibodies that specifically bind to normal SK or IK channel proteins or subsequences thereof. The antibody may be free or immobilized on a solid support such as a test tube, a microwell plate, a dipstick and the like. The kit may also contain instructional materials teaching the use of the antibody in an assay for the detection of SK or IK channel protein. The kit may contain appropriate reagents for detection of labels, positive and negative controls, washing solutions, dilution buffers and the like.

Assays for Compounds that Increase or Decrease K⁺ Flux

Isolated SK or IK channel nucleic acids of the present invention which are expressed in cells can be used in a variety of assays to detect compounds that increase or decrease the flux (i.e., influx or efflux) of potassium through the SK or IK channels, respectively. Generally, compounds that decrease potassium ion flux will cause a decrease by at least 10% or 20%, more preferably by at least 30%, 40%, or 50%, and most preferably by at least 70%, 80%, 90% or 100%. Compounds that increase the flux of potassium ions will cause a

detectable increase in the potassium ion current density by increasing the probability of a SK or IK channel being open and allowing the passage of potassium ions. Typically the flux will increase by at least 20%, 50%, 100%, or 200%, often by at least 400%, 600%, 1,000%, 5,000% or 10,000%. Increased or decreased flux of potassium may be assessed by determining changes in polarization (i.e., electrical potential) of the cell expressing the SK or IK channel. A particularly preferred means to determine changes in cellular polarization is the voltage-clamp technique. Whole cell currents are conveniently determined using the conditions set forth in Example 3. Other known assays include: radiolabeled rubidium flux assays and fluorescence assays using voltage-sensitive dyes. See, e.g., Vestergaard-Bogind et al., *J. Membrane Biol.*, 88:67-75 (1988); Daniel et al., *J. Pharmacol. Meth.*, 25:185-193 (1991); Holevinsky et al., *J. Membrane Biology*, 137:59-70 (1994). Assays for compounds capable of inhibiting or increasing potassium flux through the SK channel protein can be performed by application of the compounds to a bath solution in contact with and comprising cells having an SK or IK channel of the present invention. See, e.g., Blatz et al., *Nature*, 323:718-720 (1986); Park, *J. Physiol.*, 481:555-570 (1994). Generally, the compounds to be tested are present in the range from 1 pM to 100 mM. Changes in function of the channels can be measured in the electrical currents or ionic flux, or by the consequences of changes in currents and flux.

The effects of the test compounds upon the function of the channels can be measured by changes in the electrical currents or ionic flux or by the consequences of changes in currents and flux. Changes in electrical current or ionic flux are measured by either increases or decreases in flux of cations such as potassium or rubidium ions. The cations can be measured in a variety of standard ways. They can be measured directly by concentration changes of the ions or indirectly by membrane potential or by radiolabeling of the ions. Consequences of the test compound on ion flux can be quite varied. Accordingly, any suitable physiological change can be used to assess the influence of a test compound on the channels of this invention. Changes in channel function can be measured by ligand displacement such as CTX release. When the functional consequences are determined using intact cells or animals, one can also measure a variety of effects such as transmitter release (e.g., dopamine), hormone release

(e.g., insulin), transcriptional changes to both known and uncharacterized genetic markers (e.g., northern blots), cell volume changes (e.g., in red blood cells), immuno-responses (e.g., T cell activation), changes in cell metabolism such as cell growth or pH changes.

5 Preferably, the SK channel of the assay will be selected from a channel protein of SEQ ID NOS:1, 2, 3, 4, 19, 20, 43 or 47 or conservatively modified variant thereof. An IK channel of the assay will preferably have a sequence as shown in SEQ ID NO:32, or conservatively modified variant thereof. Alternatively, the SK channel of the assay will be derived from a eukaryote and
10 include an amino acid subsequence having sequence similarity to the core region of SK channel proteins of SEQ ID NOS:1 through 4, 19, 20, 43 and/or 47. The IK will typically be derived from a eukaryote and include an amino acid subsequence having sequence similarity to the core region of IK channel proteins of SEQ ID NO:32. Generally, the functional SK or IK channel protein will be at least 400,
15 450, 500, or 550 amino acids in length. The percentage of sequence similarity with the core region of a protein selected from the group consisting of: SEQ ID NO:1, 2, 3, 4, 19, 20, 32, 43 and 47 will be any one of the integers between 60 and 100. Generally, the sequence similarity will be at least 60%, typically at least 70%, generally at least 75%, preferably at least 80%, more preferably at least
20 85%, most preferably at least 90%, and often at least 95%. Thus, SK channel homologs will hybridize, under moderate hybridization conditions, to a nucleic acid of at least 300 nucleotides in length from the core region of a nucleic acid
selected from the group consisting of SEQ ID NOS:13, 14, 15, 16, 21, 22, ^{44 and 48} and complementary sequences thereof. IK channel homologs will hybridize, under
25 moderate hybridization conditions, to a nucleic acid of at least 300 nucleotides in length from the core region of a nucleic acid such as SEQ ID NO:31.

The "core region" or "core sequence" of SEQ ID NOS:13-16, 21, 22, 44 and 48 corresponds to the encoded region of alignment between SEQ ID NOS: 1, 2, 3, 4, 19, 20, 43, and 47 with and from rSK2 (SEQ ID NO:2) amino acid
30 residue 135 to 462. The core region of hIK1 is from amino acid residue 25 through residue 351. In preferred embodiments, the SK channel will have at least 90% sequence similarity, as compared to the core sequence from a sequence of ID NO: 1, 2, 3, 4, 19, 20, 43, or 47 over a comparison window of any of from any

one of 20 contiguous amino acid residues to 300 contiguous amino acid residues from within the core region. In preferred embodiments, the IK channel will have at least 90% sequence similarity, as compared to the core sequence of SEQ ID NO:32, over a comparison window of any of from any one of 20 contiguous amino acid residues to 300 contiguous amino acid residues from within the core region.

5 The SK channel homologs will generally have substantially similar conductance characteristics (e.g., 2-60 pS) and calcium sensitivities (30 nM - 10 μ M). IK channel homologs will likewise have similar SK channels conductance characteristics as a IK channel (e.g., 20-80 pS) and calcium sensitivities (30 nM - 10 μ M). Chimeras formed by expression of at least two of ~~SEQ ID NOS: 1, 2, 3,~~
 10 a ~~SEQ ID NOS: 1, 2, 3, 4, 19, 20, 32, 43 or 47~~
 a ~~4, 19, 20, or 32~~ can also be used. In a preferred embodiment, the cell placed in contact with a compound which is assayed for increasing or decreasing potassium flux is a eukaryotic cell, more preferably an oocyte of *Xenopus* (e.g., *Xenopus laevis*).

15 Yet another assay for compounds that increase or decrease potassium flux in calcium activated potassium channels involves "virtual genetics," in which a computer system is used to generate a three-dimensional structure of SK and IK proteins based on the structural information encoded by the amino acid sequence. The amino acid sequence interacts directly and actively with a
 20 preestablished algorithm in a computer program to yield secondary, tertiary, and quaternary structural models of the protein. The models of the protein structure are then examined to identify regions of the structure that have the ability to bind to ligands. These regions are then used to identify ligands that bind to the protein.

25 The three-dimensional structural model of the protein is generated by inputting channel protein amino acid sequences or nucleic acid sequences encoding a channel protein into the computer system. The amino acid sequence of the channel protein is selected from the group consisting of: SEQ ID NOS: 1, 2, 3, 4, 19, 20, 32, 43, 47, and conservatively modified versions thereof. The amino
 30 acid sequence represents the primary sequence of the protein, which encodes the structural information of the protein. The amino acid sequence is input into the computer system from computer readable substrates that include, but are not limited to, electronic storage media (e.g., magnetic diskettes, tapes, cartridges,

and chips), optical media (e.g., CD ROM, telephone lines), addresses to internet sites, and RAM. The three-dimensional structural model of the channel protein is then generated by the interaction of the amino acid sequence and the computer system. The software is commercially available programs such as Biopolymer, Quanta, and Insight.

The amino acid sequence represents a primary structure that encodes the information necessary to form the secondary, tertiary and quaternary structure of the protein. The software looks at certain parameters encoded by the primary sequence to generate the structural model. These parameters are referred to as "energy terms," and primarily include electrostatic potential, hydrophobic potential, solvent accessible surface, and hydrogen bonding. Secondary energy terms include van der Waals potential. Biological molecules form the structures that minimize the energy terms in a cumulative fashion. The computer program is therefore using these terms encoded by the primary structure or amino acid sequence to create the secondary structural model.

The tertiary structure of the protein encoded by the secondary structure is then formed on the basis of the energy terms of the secondary structure. The user at this point can input additional variables such as whether the protein is membrane bound or soluble, its location in the body, and whether it is cytoplasmic, surface, or nuclear. These variables along with the energy terms of the secondary structure are used to form the model of the tertiary structure. In modeling the tertiary structure, the computer program matches hydrophobic protein faces of secondary structure with like, and hydrophilic secondary structure with like.

Finally, quaternary structure of multi-subunit proteins can be modeled in a similar fashion, using anisotropy terms. These terms interface different protein subunits to energetically minimize the interaction of the subunits. In the case of channel proteins, typically four identical subunits make up the quaternary structure of the channel.

Once the structure has been generated, potential ligand binding regions are identified by the computer system. Three-dimensional structures for potential ligands are generated by inputting amino acid and nucleotide sequences or chemical formulas of compounds, as described above. The three-dimensional

structure of the potential ligand is then compared to that of the channel protein to identify ligands that bind to the channel protein. Binding affinity between the protein and ligands is determined using energy terms to determine which ligands have an enhanced probability of binding to the protein.

5 Computer systems are also used to screen for mutations of SK and IK genes. Such mutations can be associated with disease states. Once the mutations are identified, diagnostic assays can be used to identify patients having such mutated genes associated with disease states. Identification of the mutated SK and IK genes involves receiving input of a first nucleic acid sequence
10 encoding a calcium channel protein having an amino acid sequence selected from the group consisting of SEQ ID NOS:1, 2, 3, 4, 20, 32, 43, 47, and conservatively modified versions thereof. The sequence is input into the computer system as described above. The first nucleic acid sequence is then compared to a second nucleic acid sequence that has substantial identity to the first nucleic acid
15 sequence. The second nucleic acid sequence is input into the computer system in the manner described above. Once the first and sequence sequences are compared, nucleotide differences between the sequences are identified. Such sequences can represent allelic differences in SK and IK genes, and mutations associated with disease states.

20 Cellular Transfection and Gene Therapy

 The present invention provides packageable SK and IK channel protein nucleic acids (cDNAs), *supra*, for the transfection of cells *in vitro* and *in vivo*. These packageable nucleic acids can be inserted into any of a number of well known vectors for the transfection of target cells and organisms as described
25 below. The nucleic acids are transfected into cells, *ex vivo* or *in vivo*, through the interaction of the vector and the target cell. The SK or IK channel protein nucleic acid, under the control of a promoter, then expresses the calcium-activated potassium channel protein of the present invention thereby mitigating the effects of absent, partial inactivation, or abnormal expression of the SK or IK channel
30 protein gene.

 Such gene therapy procedures have been used to correct acquired and inherited genetic defects, cancer, and viral infection in a number of contexts.

The ability to express artificial genes in humans facilitates the prevention and/or cure of many important human diseases, including many diseases which are not amenable to treatment by other therapies. As an example, *in vivo* expression of cholesterol-regulating genes, genes which selectively block the replication of HIV, and tumor-suppressing genes in human patients dramatically improves the treatment of heart disease, AIDS, and cancer, respectively. For a review of gene therapy procedures, see Anderson, *Science* (1992) 256:808-813; Nabel and Felgner (1993) *TIBTECH* 11: 211-217; Mitani and Caskey (1993) *TIBTECH* 11: 162-166; Mulligan (1993) *Science* 926-932; Dillon (1993) *TIBTECH* 11: 167-175; Miller (1992) *Nature* 357: 455-460; Van Brunt (1988) *Biotechnology* 6(10): 1149-1154; Vigne (1995) *Restorative Neurology and Neuroscience* 8: 35-36; Kremer and Perricaudet (1995) *British Medical Bulletin* 51(1) 31-44; Haddada et al. (1995) in *Current Topics in Microbiology and Immunology* Doerfler and Böhm (eds) Springer-Verlag, Heidelberg Germany; and Yu et al., *Gene Therapy* (1994) 1:13-26.

Delivery of the gene or genetic material into the cell is the first critical step in gene therapy treatment of disease. A large number of delivery methods are well known to those of skill in the art. Such methods include, for example liposome-based gene delivery (Debs and Zhu (1993) WO 93/24640; Mannino and Gould-Fogerite (1988) *BioTechniques* 6(7): 682-691; Rose U.S. Pat No. 5,279,833; Brigham (1991) WO 91/06309; and Felgner et al. (1987) *Proc. Natl. Acad. Sci. USA* 84: 7413-7414), and replication-defective retroviral vectors harboring a therapeutic polynucleotide sequence as part of the retroviral genome (see, e.g., Miller et al. (1990) *Mol. Cell. Biol.* 10:4239 (1990); Kolberg (1992) *J. NIH Res.* 4:43, and Cornetta et al. *Hum. Gene Ther.* 2:215 (1991)). Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SIV), human immuno deficiency virus (HIV), and combinations thereof. See, e.g., Buchscher et al. (1992) *J. Virol.* 66(5) 2731-2739; Johann et al. (1992) *J. Virol.* 66 (5):1635-1640 (1992); Sommerfelt et al., (1990) *Virol.* 176:58-59; Wilson et al. (1989) *J. Virol.* 63:2374-2378; Miller et al., *J. Virol.* 65:2220-2224 (1991); Wong-Staal et al., PCT/US94/05700, and Rosenberg and Fauci (1993) in *Fundamental Immunology*,

Third Edition Paul (ed) Raven Press. Ltd., New York and the references therein, and Yu *et al.*, *Gene Therapy* (1994) *supra*).

AAV-based vectors are also used to transduce cells with target nucleic acids, e.g., in the *in vitro* production of nucleic acids and peptides, and in *in vivo* and *ex vivo* gene therapy procedures. See, West *et al.* (1987) *Virology* 160:38-47; Carter *et al.* (1989) U.S. Patent No. 4,797,368; Carter *et al.* WO 93/24641 (1993); Kotin (1994) *Human Gene Therapy* 5:793-801; Muzyczka (1994) *J. Clin. Invest.* 94:1351 and Samulski (*supra*) for an overview of AAV vectors. Construction of recombinant AAV vectors are described in a number of publications, including Lebkowski, U.S. Pat. No. 5,173,414; Tratschin *et al.* (1985) *Mol. Cell. Biol.* 5(11):3251-3260; Tratschin, *et al.* (1984) *Mol. Cell. Biol.*, 4:2072-2081; Hermonat and Muzyczka (1984) *Proc. Natl. Acad. Sci. USA*, 81:6466-6470; McLaughlin *et al.* (1988) and Samulski *et al.* (1989) *J. Virol.*, 63:03822-3828. Cell lines that can be transfected by rAAV include those described in Lebkowski *et al.* (1988) *Mol. Cell. Biol.*, 8:3988-3996.

A. Ex vivo transfection of Cells

Ex vivo cell transfection for diagnostics, research, or for gene therapy (e.g., via re-infusion of the transfected cells into the host organism) is well known to those of skill in the art. In a preferred embodiment, cells are isolated from the subject organism, transfected with an SK or IK channel protein nucleic acid (gene or cDNA), and re-infused back into the subject organism (e.g., patient). Various cell types suitable for *ex vivo* transfection are well known to those of skill in the art (see, e.g., Freshney *et al.*, *Culture of Animal Cells, a Manual of Basic Technique*, third edition Wiley-Liss, New York (1994)) and the references cited therein for a discussion of how to isolate and culture cells from patients).

As indicated above, in a preferred embodiment, the packageable nucleic acid which encodes an SK or IK channel protein is under the control of an activated or constitutive promoter. The transfected cell(s) express a functional SK or IK channel protein which mitigates the effects of deficient or abnormal SK or IK channel protein gene expression.

In one particularly preferred embodiment, stem cells are used in *ex vivo* procedures for cell transfection and gene therapy. The advantage to using

stem cells is that they can be differentiated into other cell types *in vitro*, or can be introduced into a mammal (such as the donor of the cells) where they will engraft in the bone marrow. Methods for differentiating CD34⁺ cells *in vitro* into clinically important immune cell types using cytokines such as GM-CSF, IFN- γ and TNF- α are known (see, Inaba *et al.* (1992) *J. Exp. Med.* 176, 1693-1702, and Szabolcs *et al.* (1995) 154: 5851-5861).

Stem cells are isolated for transduction and differentiation using known methods. For example, in mice, bone marrow cells are isolated by sacrificing the mouse and cutting the leg bones with a pair of scissors. Stem cells are isolated from bone marrow cells by panning the bone marrow cells with antibodies which bind unwanted cells, such as CD4⁺ and CD8⁺ (T cells), CD45⁺ (panB cells), GR-1 (granulocytes), and Ia^d (differentiated antigen presenting cells). For an example of this protocol see, Inaba *et al.* (1992) *J. Exp. Med.* 176, 1693-1702.

In humans, bone marrow aspirations from iliac crests are performed *e.g.*, under general anesthesia in the operating room. The bone marrow aspirations is approximately 1,000 ml in quantity and is collected from the posterior iliac bones and crests. If the total number of cells collected is less than about 2×10^8 /kg, a second aspiration using the sternum and anterior iliac crests in addition to posterior crests is performed. During the operation, two units of irradiated packed red cells are administered to replace the volume of marrow taken by the aspiration. Human hematopoietic progenitor and stem cells are characterized by the presence of a CD34 surface membrane antigen. This antigen is used for purification, *e.g.*, on affinity columns which bind CD34. After the bone marrow is harvested, the mononuclear cells are separated from the other components by means of ficol gradient centrifugation. This is performed by a semi-automated method using a cell separator (*e.g.*, a Baxter Fenwal CS3000+ or Terumo machine). The light density cells, composed mostly of mononuclear cells are collected and the cells are incubated in plastic flasks at 37°C for 1.5 hours. The adherent cells (monocytes, macrophages and B-Cells) are discarded. The non-adherent cells are then collected and incubated with a monoclonal anti-CD34 antibody (*e.g.*, the murine antibody 9C5) at 4°C for 30 minutes with gentle rotation. The final concentration for the anti-CD34 antibody is 10 μ g/ml.

After two washes, paramagnetic microspheres (Dyna Beads, supplied by Baxter Immunotherapy Group, Santa Ana, California) coated with sheep antimouse IgG (Fc) antibody are added to the cell suspension at a ratio of 2 cells/bead. After a further incubation period of 30 minutes at 4°C, the rosetted cells with magnetic beads are collected with a magnet. Chymopapain (supplied by Baxter Immunotherapy Group, Santa Ana, California) at a final concentration of 200 U/ml is added to release the beads from the CD34+ cells. Alternatively, and preferably, an affinity column isolation procedure can be used which binds to CD34, or to antibodies bound to CD34 (see, the examples below). See, Ho *et al.* (1995) *Stem Cells* 13 (suppl. 3): 100-105. See also, Brenner (1993) *Journal of Hematotherapy* 2: 7-17.

In another embodiment, hematopoietic stem cells are isolated from fetal cord blood. Yu *et al.* (1995) *Proc. Natl. Acad. Sci. USA*, 92: 699-703 describe a preferred method of transducing CD34+ cells from human fetal cord blood using retroviral vectors.

B. In vivo Transfection

Vectors (e.g., retroviruses, adenoviruses, liposomes, etc.) containing therapeutic nucleic acids can be administered directly to the organism for transduction of cells *in vivo*. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells. The packaged nucleic acids are administered in any suitable manner, preferably with pharmaceutically acceptable carriers. Suitable methods of administering such packaged nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present invention.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the packaged nucleic acid suspended in

diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, tragacanth, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art.

The packaged nucleic acids, alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Suitable formulations for rectal administration include, for example, suppositories, which consist of the packaged nucleic acid with a suppository base. Suitable suppository bases include natural or synthetic triglycerides or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the packaged nucleic acid with a base, including, for example, liquid triglycerides, polyethylene glycols, and paraffin hydrocarbons.

Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions can be administered,

for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. Parenteral administration and intravenous administration are the preferred methods of administration. The formulations of packaged nucleic acid can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials.

Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Cells transduced by the packaged nucleic acid as described above in the context of *ex vivo* therapy can also be administered intravenously or parenterally as described above.

The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time. The dose will be determined by the efficacy of the particular vector employed and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular vector, or transduced cell type in a particular patient.

In determining the effective amount of the vector to be administered in the treatment or prophylaxis of conditions owing to diminished or aberrant expression of SK or IK channel protein, the physician evaluates circulating plasma levels of the vector, vector toxicities, progression of the disease, and the production of anti-vector antibodies. In general, the dose equivalent of a naked nucleic acid from a vector is from about 1 μg to 100 μg for a typical 70 kilogram patient, and doses of vectors which include a retroviral particle are calculated to yield an equivalent amount of therapeutic nucleic acid.

For administration, inhibitors and transduced cells of the present invention can be administered at a rate determined by the LD-50 of the inhibitor, vector, or transduced cell type, and the side-effects of the inhibitor, vector or cell type at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses.

In a preferred embodiment, prior to infusion, blood samples are obtained and saved for analysis. Between 1×10^8 and 1×10^{12} transduced cells are infused intravenously over 60- 200 minutes. Vital signs and oxygen saturation

by pulse oximetry are closely monitored. Blood samples are obtained 5 minutes and 1 hour following infusion and saved for subsequent analysis. Leukopheresis, transduction and reinfusion can be repeated every 2 to 3 months. After the first treatment, infusions can be performed on an outpatient basis at the discretion of the clinician. If the reinfusion is given as an outpatient, the participant is monitored for at least 4, and preferably 8 hours following the therapy.

Transduced cells are prepared for reinfusion according to established methods. See, Abrahamsen *et al.* (1991) *J. Clin. Apheresis*, 6: 48-53; Carter *et al.* (1988) *J. Clin. Arpheresis*, 4:113-117; Aebersold *et al.* (1988) *J. Immunol. Meth.*, 112: 1-7; Muul *et al.* (1987) *J. Immunol. Methods*, 101:171-181 and Carter *et al.* (1987) *Transfusion* 27: 362-365. After a period of about 2-4 weeks in culture, the cells should number between 1×10^8 and 1×10^{12} . In this regard, the growth characteristics of cells vary from patient to patient and from cell type to cell type. About 72 hours prior to reinfusion of the transduced cells, an aliquot is taken for analysis of phenotype, and percentage of cells expressing the therapeutic agent.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Example 1

Example 1 describes the isolation and sequencing of clones encoding small and intermediate conductance, calcium-dependent potassium channels.

A. Small conductance potassium channels, with the exception of the minK protein (Takumi *et al.*, *Science*, 242:1042-1045 (1988), share a common structural motif within the pore region including the sequence which dictates the characteristic selectivity sequence for monovalent cations (Heginbotham *et al.*, *Biophys. J.*, 66:1061-1067 (1994)).

A BLAST search of the EST database using the query sequence FXSIPXXXWWAXVTMTTVGYGDMXP (SEQ ID NO:45), allowing for mismatches, retrieved known potassium channel sequences and Genbank #M62043.

Oligonucleotides corresponding to nucleotides 6-36 (sense) and 258-287

5 (antisense) of #M62043 were synthesized (Genosys, The Woodlands, TX), radiolabeled using polynucleotide kinase (BRL) and ^{32}P -ATP (NEN), and used to screen $\sim 10^6$ recombinant phage from the human hippocampal cDNA library (40 % formamide; 1 M NaCl, 1 % SDS, 37°C; washed at 1 X SSC, 50°C). Double positively hybridizing phage were purified by rescreening at reduced densities.

10 cDNA inserts were subcloned into M13 and the nucleotide sequences determined using the dideoxy chain termination method and T7 DNA polymerase (Sequenase, UBI). A fragment of this clone containing the pore domain (amino acids 325-522) was radiolabeled using random primers (Boehringer) and used to screen a rat brain cDNA library (30% formamide, 1 M NaCl, 1% SDS, 37 °C; washed at 2 X

15 SSC, 50 °C). Positively hybridizing phage were purified and the nucleotide sequences of the inserts determined. Computer analyses were performed using the GCG software suite (Genetics Computer Group; version 8.1).

In addition to known potassium channels, one of the detected sequences from human hippocampus suggested it may contain the consensus motif, but included several ambiguities (Genbank #M62043). Based upon this

20 sequence, oligonucleotides were synthesized having the sequence represented by nucleotides 6 to 36 of the sense strand; and nucleotides 258 to 287 of the antisense strand. The oligonucleotides were used to probe a human hippocampal cDNA library.

25 A full length coding sequence, hSK1 (SEQ ID NO:13), was isolated and analyzed for open reading frames, Kozak consensus sequences, potential transmembrane domains, and predicted protein structure. A fragment containing the putative pore region was radiolabelled by random priming and subsequently used to probe a rat brain cDNA library using a hybridization solution of 40%

30 formamide, 1 M NaCl, 1% SDS, and 100 $\mu\text{g/ml}$ yeast RNA, at 37°C and washed using 0.5 X SSC at 55°C. Two clones containing different full length coding sequences were isolated and analyzed: rSK2 (SEQ ID NO:15), and rSK3 (SEQ ID

NO:16). In addition, a partial clone was identified representing the rat homolog of hSK1 (rSK1 (SEQ ID NO:14)).

5 The sequences predict proteins of 561 amino acids for hSK1 (SEQ ID NO:1), 580 amino acids for rSK2 (SEQ ID NO:2), and 553 amino acids for rSK3 (SEQ ID NO:3) which contain no stretches of homology (i.e., no signal above background under low stringency conditions) with other cloned potassium channels apart from a 12 amino acid sequence in the putative pore region. Hydrophobicity analysis predicts six transmembrane segments with the N- and C-termini residing inside the cell. The sequences are highly conserved across their
10 transmembrane cores (80-90% identity), but diverge in sequence and length within their N- and C-terminal domains (Table 1).

TABLE 1

FSK2MS	SCRYNNGVHR	PLSNLSSRR	NLHEMDSEAO
FSK3MS	SCKYSGGVMK	PLSRLSASRR	NLIEAEPEGO
FSK1
HSK1	MPGPRAACSE	PNPCTQVVMN	SHSYNGSVGR	P...LGSGPG ALGRDPPOPE
FSK2	PLQPPASVVG	GGGGASSPSA	AAAASSSAPE	IVVSKPEHNN SNNLALYGTG
FSK3	PLQLF.....SFSNPPE	IIISSREDNH AHQTLLHHPN
FSK1
HSK1	AGHPPQPPHS	PGLQVVVAKS	EPARPSPGSP	RGQPQDQDD EDDEEDEAGR
FSK2	GGGSTGGGGG	GGGGGGGSGH	GSSSGTKSSK	KKNQONIGYKL GHRRALFEKR
FSK3	ATHNHQHAGT	TAGSTTFP..KANK	RKNONIGYKL GHRRALFEKR
FSK1S	GKPPTVSHRL GHRRALFEKR
HSK1	QR.....AS	GKPSNVGHRL GHRRALFEKR
FSK2	KRLSDYALIF	GMFGIVVMVI	ETELSWGAYD	KASLYSLALK CLISLSTIIL
FSK3	KRLSDYALIF	GMFGIVVMVI	ETELSHGLYS	KDSMFSLALK CLISLSTIIL
FSK1	KRLSDYALIF	GMFGIVVMVT	ETELSWGVT	KESLCSFALK CLISLSTVIL
HSK1	KRLSDYALIF	GMFGIVVMVT	ETELSWGVT	KESLYSFALK CLISLSTAIL
FSK2	LGLIIVYHAR	EIQLFHVDNG	ADDWRIAMTY	ERIFFICLEI LVCAIHPVPG
FSK3	LGLIAYHTR	EVQLFVIDNG	ADDWRIAMTY	ERILYISLEM LVCAIHPVPG
FSK1	LGLVILYHAR	EIQLFVLDNG	ADDWRIAMTH	ERVSLISLEL AVCAIHPVPG
HSK1	LGLVVLHAR	EIQLFHVDNG	ADDWRIAMTC	ERVFLISLEL AVCAIHPVPG
FSK2	NYTFTWTARL	AFSYAPSTTT	ADVDIILSIP	MFLRLYLIAR VMLLHSKLFT
FSK3	EYKFFWTARL	AFSYTPSRAE	ADVDIILSIP	MFLRLYLIAR VMLLHSKLFT
FSK1	HYRFTWTARL	AFSLVPSAAE	ADVDVLLSIP	MFLRLYLLAR VMLLHSRIFT
HSK1	HYRFTWTARL	AFTYAPSVAE	ADVDVLLSIP	MFLRLYLLGR VMLLHSKIFT
FSK2	DASSRSIGAL	NKINFNTRETV	MKTLMTICPG	TVLLVFSISL WIIAAWTVRA
FSK3	DASSRSIGAL	NKINFNTRETV	MKTLMTICPG	TVLLMFSISL WIIAAWTVRV
FSK1	DASSRSIGAL	NRVTFNTRETV	TKTLMTICPG	TVLLVFSISS WIIAAWTVRV
HSK1	DASSRSIGAL	NKITFNTRETV	MKTLMTICPG	TVLLVFSISS WIIAAWTVRV
FSK2	CERYHDQODV	TSNFLGAMWL	ISITFLSIGY	GDMVPNTYCG KGVCLLTGIM
FSK3	CERYHDQODV	TSNFLGAMWL	ISITFLSIGY	GDMVPHTYCG KGVCLLTGIM
FSK1	CERYHDKQEV	TSNFLGAMWL	ISITFLSIGY	GDMVPHTYCG KGVCLLTGIM
HSK1	CERYHDKQEV	TSNFLGAMWL	ISITFLSIGY	GDMVPHTYCG KGVCLLTGIM
FSK2	GAGCTALVVA	VVARKLELTK	AEKHVHNFMM	DTQTKRVKN AAANVLRETH
FSK3	GAGCTALVVA	VVARKLELTK	AEKHVHNFMM	DTQTKRIKN AAANVLRETH
FSK1	GAGCTALVVA	VVARKLELTK	AEKHVHNFMM	DTQTKRVKN AAANVLRETH
HSK1	GAGCTALVVA	VVARKLELTK	AEKHVHNFMM	DTQTKRVKN AAANVLRETH
FSK2	LIYKNTKLVK	KIDHAKVRKH	QRKFLQAIHQ	...LRSVKME QRKLNDQANT
FSK3	LIYKHTKLLK	KIDHAKVRKH	QRKFLQAIHQ	...LRGVKME QRKLSDOANT
FSK1	LIYKHTRLVK	KPDQSRVRKH	QRKFLQAIHQ	AOKLRTVKIE QGKVNDQANT
HSK1	LIYKHTRLVK	KPDQARVRKH	QRKFLQAIHQ	AOKLRSVKIE QGKLNDQANT
FSK2	LVDLAKTONI	MYDMISDLNE	RSEDFEKRIV	TLETKLETLI GSIHALPGLI
FSK3	LVDLSKMONV	MYDLITELND	RSEDEKQIG	SLESKLEHLT ASFNSLPPLI
FSK1	LADLAKAQSI	AYEVVSELOA	QOEELEARLA	ALESRLDVLG ASLOALPSLI
HSK1	LTDLAKTOTV	MYDLVSELHA	QHEELEARLA	TLESRLDALG ASLOALPGLI
FSK2	SQTI...RQ	QORDFIETOM	ENYDKHVTYN	AERSRSSRR RRSSTAPPT
FSK3	ADTLROQQQQ	LLTAFVEARG	ISVAVG....	...TSHAPPS
FSK1	AQAICPLPPP	W...PGPSHL	TTAAQSPQSH	WLPTTASDCG *.....

hSK1 AQAIRPPPPP LPPRPGPGPQ DQAARSSPCR WTPVAPSDCG *.....
rSK2 SSES.....
rSK3 DSPIGISSTS FPEFLIF*
rSK1
hSK1

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The fourth predicted membrane spanning domain contains 3 positively charged residues that do not occupy every third position as in voltage-dependent potassium channels (Durell et al., *Biophys. J.*, 62:238-250 (1992)), but are separated by 6 and 7 residues. There are multiple consensus targets for phosphorylation by a variety of protein kinases. Some of these sites are found in all clones. However, each clone contains potential phosphorylation sites not conserved among all members. There are no conserved N-linked glycosylation sites (NXXS/T) (SEQ ID NO:46) in predicted extracellular domains, and no consensus nucleotide or calcium binding domains (E-F hands).

Northern blots of rat brain and skeletal muscle showed that rSK3 transcripts from these tissues encoded proteins that were N-terminally extended relative to the rSK3 clone SEQ ID NO:16. The nucleic acid encoding the rSK3 N-terminal extension was cloned and sequenced, and the cDNA encoding N-terminal extended rSK3 is represented by SEQ ID NO:44. In addition, endogenous rSK3 was shown to have a nucleotide sequence that encodes a protein having a C-terminus with the last 5 amino acids of SEQ ID NO:3 replaced by the last 9 amino acids of SEQ ID NO:43. Similarly, hSK3 was shown to have an N-terminal extension, and the cDNA encoding this N-terminal extension is represented by SEQ ID NO:48.

B. To isolate intermediate conductance calcium activated K^+ proteins, one can use PCR under standard conditions. Suitable primers are SEQ ID NOS:34 and 35 which yield a probe of about 270 bases and SEQ ID NOS:36 and 37 which yield a probe of about 165 bases. These primers can be used to amplify plasmid DNA comprising cloned hIK1 or on reverse transcribed RNA from a tissue which expresses hIK1, such as a cDNA library from pancreas. The PCR reaction will yield DNA fragments of the specified size which contain sequences specific to hIK1 and related genes. These DNA fragments are subsequently labeled for use as hybridization probes by standard random-priming protocols. The labeled probes are then used to screen libraries at high stringency to isolate only hIK1 sequences, or at moderately low stringency (30-40% formamide, 37°C hyb/ 1xSSC, 55°C wash) to isolate putatively related sequences. Alternatively,

one can amplify the intact hIK1 gene from a pancreas cDNA library using PCR primer pair SEQ ID NOS:38 and 39 or 40 and 41.

Example 2

5 Example 2 describes *in situ* hybridization of rat brain sections using sequences distinct for each of the rat SK channel clones, and determination of transcript sizes from various peripheral tissues.

Care and handling of adult female Sprague-Dawley rats were in accordance with the highest standards of institutional guidelines. Rats were deeply anesthetized with pentobarbital and perfused transcardially with ice-cold saline, followed by ice-cold 4% paraformaldehyde in 0.1 M sodium borate (pH 10 9.5). The brains were removed quickly and post-fixed overnight at 4 °C in 4% paraformaldehyde in borate buffer (pH 9.5) containing 10% sucrose. Cryostat microtome sections (25 mm) were mounted onto gelatin- and poly-L-lysine-coated glass slides and incubated for 15 min in 4% paraformaldehyde in 0.1 M PBS, 15 washed twice in 0.1 M PBS, and treated for 30 min at 37 °C in 10 mg/ml proteinase K in 100 mM Tris, 50 mM EDTA (pH 8), followed by 0.0025% acetic anhydride in 0.1 M triethanolamine at room temperature. The sections were then washed in 2 X SSC, dehydrated in increasing concentrations of ethanol, and vacuum-dried at room temperature.

20 Templates for probe synthesis represented C-terminal and 3' untranslated sequences unique to each of the clones, and were subcloned into pKS. Using linearized template DNA, ³⁵S -labeled antisense cRNA probe heated to 65 °C for 5 min and diluted to 10⁷ cpm/ml in hybridization buffer; 66 % formamide, 260 mM NaCl, 1.3 X Denhardt solution, (13 mM Tris, pH 8.0, 1.3 mM 25 EDTA, 13 % dextran sulfate). Sections in hybridization mixture were covered with siliconized glass coverslips and sealed using DPX mountant. After incubating at 58 °C for 20 hr, the slides were soaked in 4 X SSC to remove coverslips, then rinsed in 4 X SSC (4 times, 5 min each) prior to ribonuclease A treatment (20 mg/ml for 30 min at 37 °C). The slides were then rinsed in decreasing 30 concentrations of SSC containing 1 mM DTT to a final stringency of 0.1 X SSC, 1 mM DTT for 30 min at 65 °C. After dehydrating the sections in increasing concentrations of ethanol, they were vacuum-dried and exposed to DuPont

Cronex-4 X-ray film for 7 days. The film was scanned by a Microtek ScanMaker 1850S at 728 pixel/cm resolution and the images analyzed using Image v1.55 software (NIH) and Photoshop (Adobe).

5 The results indicate that mRNAs to the rat sequences are broadly distributed throughout the CNS, in characteristic but overlapping patterns. rSK1 is expressed in the hippocampus and the dentate gyrus, the granular layer of the cerebellum, and the anterior olfactory nucleus. rSK1 mRNA was also detected in the subiculum, the olfactory tubercle, and the neocortex. rSK2 mRNA is the most widely expressed, with highest expression in the hippocampus and lower levels in 10 the dentate gyrus, the olfactory bulb and the anterior olfactory nucleus. rSK2 mRNA was also detected in the granular layer of the cerebellum, the reticular nucleus of the thalamus, and the pontine nucleus. The pattern of *in situ* hybridization for rSK2 mRNA is coincident with the pattern of radiolabeled apamin binding in rat brain (Gelhart, *Neuroscience*, 52:191-205 (1993)). rSK3 mRNA was 15 detected in the olfactory tubercle and olfactory bulb, throughout the thalamus, the lateral septum, the ventral tegmental area, and the substantia nigra pars compacta. Moderate levels were detected throughout the hypothalamus, the caudate putamen, and the nucleus accumbens.

20 The same distinct sequences for rSK1 and rSK2 were used to probe Northern blots prepared with mRNA isolated from total brain and several peripheral tissues. Total RNA was extracted (Chirgwin et al., *Biochem.*, 18:5294-5300 (1979)) from rat brain, adrenal gland, thymus, spleen, skeletal muscle, heart, kidney, liver, and lung of 3 week old Sprague-Dawley rats. Poly (A)⁺ mRNA was purified by oligo d(T) cellulose chromatography (Collaborative Research), and 25 3 μ g from each tissue was prepared as a Northern blot by electrophoresis through a 1 % agarose-formaldehyde gel and transfer to Genescreen (NEN) nylon membranes. Antisense riboprobes of the same sequence as used for *in situ* hybridization were synthesized from linearized DNA templates using ³²P-UTP (NEN). Blots were hybridized in 50% formamide, 5 % SDS, 400 mM NaPO₄, 1 30 mM EDTA at 60 °C for 12 hours, followed by washes in 0.05 X SSC at 65 °C, and visualized using a Phosphorimager 445 SI (Molecular Dynamics) after 15 hours.

rSK1 mRNA was detected in rat brain and heart, while rSK2 mRNA was detected in brain and adrenal gland. The results show that rSK1 mRNAs of

different sizes are present in brain (3.2 kb) and heart (4.4 kb). rSK2 mRNA was detected in brain and adrenal gland as two bands of 2.2 and 2.4 kb. Neither rSK1 nor rSK2 mRNA was detected from lung, liver, kidney, thymus, spleen, or skeletal muscle.

Example 3

Example 3 describes *in vitro* expression of SK and IK channel proteins.

3A. Example 3A describes *in vitro* expression of rSK2 and hSK1 mRNAs in *Xenopus* oocytes and measurements of electrical conductance.

In vitro mRNA synthesis and oocyte injections were performed as described in Adelman et al., *Neuron*, 9:209-216 (1992). *Xenopus* care and handling were in accordance with the highest standards of institutional guidelines. Frogs underwent no more than two surgeries, separated by at least three weeks, and surgeries were performed using well established techniques. Frogs were anesthetized with an aerated solution of 3-aminobenzoic acid ethyl ester.

Oocytes were studied 2-5 days after injection with 2 ng of mRNA. Whole cell currents were measured after mRNA injection using a two electrode voltage clamp with a CA-1 amplifier interfaced to a Macintosh Quadra 650 computer. Data were simultaneously acquired through Pulse (Heka, Germany) at 500 Hz and Chart (AD Instruments, Australia) at 10 Hz. During recording, oocytes were continuously superfused with ND-96 solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES (pH 7.5 with NaOH) at room temperature. To minimize Cl⁻ currents, some oocytes were soaked and studied in Cl⁻-free ND96 solution (96 mM Nagluconate, 2 mM Kgluconate, 2.7 mM Cagluconate, 1 mM Mggluconate, 5 mM HEPES, pH 7.5 with NaOH). Voltage protocols from a holding potential of -80mV failed to evoke currents different from control oocytes.

Because the expression pattern of rSK2 is similar to that of mGluR1a, a metabotropic glutamate receptor (Houamed et al., *Science*, 252:1318-1321 (1991); Masu et al., *Nature*, 349:760-765 (1991)), mGluR1a mRNA was injected with or without the SK mRNAs. Addition of glutamate (1 mM)

to the bath comprising the oocyte injected with mGluR1a mRNA alone evoked a transient inward current due to activation of endogenous calcium-activated chloride channels following the release of intracellular calcium (Houamed et al., *Science*, 252:1318-1321 (1991); Masu et al., *Nature*, 349:760-765 (1991)).

5 Similar results were obtained in six other oocytes injected with mGluR1a. Voltage ramps from -120 to 60 mV applied near the peak of the inward response evoked an outwardly rectifying current that reversed at -25 mv, near the Cl⁻ reversal potential. Addition of glutamate (1 mM) to oocytes coinjected with mGluR1a and rSK2 mRNA evoked the transient calcium-activated chloride current observed with
10 mGluR1a injected oocytes, followed by a large transient outward current. Similar results were obtained in 14 other oocytes coinjected with mGluR1a and rSK2. Voltage ramps from -120 to 60 mV applied near the peak of the outward response evoked a large inwardly rectifying current that reversed near -95 mV, close to the K⁺ reversal potential. This result was obtained with each of the cloned subunits
15 and suggested that the cloned sequences encode potassium channels.

Following establishment of the 2-electrode voltage clamp, the oocyte was impaled with a third electrode containing 200 mM EGTA, pH adjusted to 7.2 with KOH. The input resistance was monitored during impalement to insure oocyte viability. At the indicated time, 50 nl of the EGTA solution was injected
20 into the oocyte. Assuming an oocyte volume of 1 μ l, the predicted final concentration of EGTA was 10 mM. Intracellular injection of EGTA abolished both current responses evoked by subsequent application of glutamate indicating that both components are calcium-activated. Similar results were obtained in 3 other oocytes coinjected with mGluR1a and rSK2. Current-voltage relation of
25 oocytes injected with rSK2 mRNA in Cl⁻-free external solution containing 2, 6 or 20 mM K⁺. The current was activated by injection of CaCl₂ to a final concentration of ~1 mM (Adelman et al., *Neuron*, 9:209-216 (1992)). Background current was determined by application of 100 nM apamin. The apamin-insensitive background current did not vary with external K⁺.

30 Two days after injection, the oocytes were soaked for >24 hours in Cl⁻ free ND96 solution to minimize Cl⁻ currents. In the 2-electrode recording mode, the channel was activated by injection of 5 nl of 200 mM CaCl₂ through a third electrode resulting in a final intracellular concentration of ~1 mM Ca²⁺. This

procedure resulted in a longer lasting activation of the K^+ current than that activated by glutamate in oocytes coinjected with mGluR1a and rSK2. In these oocytes, the reversal potential was determined relative to background current in 100 nM apamin. The mean reversal potential \pm S.D. plotted versus $[K^+]_o$ yields a slope of 55.4 mV/decade change in $[K^+]_o$ and a y-intercept of -110 mV at 1 mM $[K^+]_o$.

Macroscopic currents were also recorded from excised patches. Currents were elicited by 2.5 second voltage ramps from -100 to 100 mV in an excised inside-out patch from an oocyte expressing rSK2. Without bath applied calcium, currents were not different from control oocytes. Oocytes were injected as described for two-electrode voltage clamp recordings.

Two to nine days after injection, inside-out macropatches were excised into a bath solution containing 116 mM Kgluconate, 4 mM KCl, 10 mM HEPES (pH 7.25, adjusted with KOH) supplemented with $CaCl_2$ and/or EGTA. To obtain nominally Ca-free solution, 1 mM EGTA was added. Alternatively, $CaCl_2$ was added to the bath solution to give free calcium concentrations of 1-10 μ M. In this case, the proportion of calcium binding to gluconate was determined by a computer program (CaBuf) assuming a stability constant for Ca^{2+} gluconate of $15.9M^{-1}$ (Dawson et al., *Data for Biochemical Research* (Oxford University Press, New York, (1969)). To obtain Ca^{2+} concentrations below 1 μ M, 5 mM EGTA was added to the bath solution and $CaCl_2$ was added as calculated using the CaBuf program and published stability constants (Fabiato et al., *J. Physiol.*, 75:463-505 (1979)). For experiments in which Mg^{2+} was added to the bath solution, $MgCl_2$ was added to the total concentrations stated in the text. Under these conditions, binding of Mg^{2+} to gluconate is negligible (stability constant $1.7 M^{-1}$).

Electrodes were pulled from thin-walled, filamented borosilicate glass (World Precision Instruments) and filled with 116 mM Kgluconate, 4 mM KCl, 10 mM HEPES (pH 7.25). Electrode resistance was typically 2-5 M Ω . Membrane patches were voltage clamped using an Axopatch 200A amplifier (Axon Instruments). The data were low-pass Bessel filtered at 2 kHz and acquired using Pulse software (HEKA Elektronik). Analysis was performed using Pulse, Kaleidograph (Abelbeck), or IGOR (Wavemetrics) software. All experiments were performed at room temperature from a holding potential of -80 mV. 2.5 second

voltage ramps from -100 to 100 mV were acquired at a sampling frequency of 500 Hz. Alternatively, current-voltage relationships were obtained from the mean current during 500 ms commands to voltages between -100 and 100 mV in 20 mV increments, sampled at 5 kHz.

5 Addition of 5 μM Ca^{2+} to the intracellular (bath) solution evoked a substantial current. Voltage ramps in symmetrical 120 mM K^+ and in the absence of internal Mg^{2+} revealed a current-voltage relationship with slight inward rectification. Voltage steps between -100 and 100 mV, from a holding potential of -80 mV, evoked time-independent currents. The derived I-V relationship reflects
10 the inward rectification apparent from voltage ramps. The current was evoked by voltage steps from an inside-out macropatch excised from an oocyte expressing rSK2. With 5 μM Ca^{2+} in the bath, the membrane was stepped from a holding potential of -80 mV to test potentials between -100 and 100 mV and then repolarized to -50 mV. Currents activated instantaneously and showed no
15 inactivation during the 500 ms test pulses. Similar results were obtained for hSK1, except that the inward rectification was not as pronounced. These results identify this new family as calcium-activated potassium channels.

3B. Example 3B describes the electrophysiology of the hIK1 channel. All hIK1 channel subunits were subcloned into the oocyte expression
20 vector pBF (unpublished, graciously provided by Dr. B. Fakler) which provides 5' and 3' untranslated regions from the *Xenopus* β -globin gene flanking a polylinker containing multiple restriction sites. *In vitro* mRNAs were generated using SP6 polymerase (GibcoBRL); following synthesis, mRNAs were evaluated spectrophotometrically and by ethidium bromide staining after agarose gel
25 electrophoresis.

As described above, *Xenopus* care and handling were in accordance with the highest standards of institutional guidelines. Frogs underwent no more than two surgeries, separated by at least three weeks, and All surgeries were performed using well established techniques. Frogs were anesthetized with an
30 aerated solution of 3-aminobenzoic acid ethyl ester. Oocytes were studied 2-14 days after injection with 0.5-5 ng of mRNA.

Inside-out macropatches were excised into an intracellular solution containing 116 mM K-gluconate, 4 mM KCl, 10 mM HEPES (pH 7.2, adjusted with KOH) supplemented with CaCl_2 to give free calcium concentration of 5 μM ; the proportion of calcium binding to gluconate was determined by a computer program (CaBuf) assuming a stability constant for Ca^{2+} gluconate of 15.9M^{-1} (Dawson *et al.*, 1969). To obtain Ca^{2+} concentrations below 1 μM , 1 mM EGTA was added to the bath solution and CaCl_2 was added as calculated using the CaBuf program and published stability constants (Fabiato and Fabiato, 1979). Electrodes were pulled from thin-walled, filamented borosilicate glass (World Precision Instruments) and filled with 116 mM K-gluconate, 4 mM KCl, 10 mM HEPES (pH 7.2). Electrode resistance was typically 2-5 $\text{M}\Omega$. For outside-out macropatches, the solutions were reversed. Membrane patches were voltage clamped using an Axopatch 200A amplifier (Axon Instruments). The data were low-pass Bessel filtered at 1 kHz and acquired using Pulse software (HEKA Elektronik). Analysis was performed using Pulse, Kaleidagraph (Abelbeck), or IGOR (Wavemetrics) software. Unless otherwise stated all experiments were performed at room temperature from a holding potential of 0 mV. 2.5 second voltage ramps from -100 to either 60 or 100 mV were acquired at a sampling frequency of 500 Hz. Values were expressed as mean \pm SD. Statistical differences were determined using an unpaired t-test; p values <0.05 were considered significant.

For single channel recordings, oocytes were bathed in 116 mM Kgluconate, 4 mM KCl, 10 mM HEPES, 5 mM EGTA, pH 7.2 adjusted with CaCl_2 to yield the reported concentration of free Ca^{2+} . All recordings were performed in the inside-out patch configuration using thick-walled quartz electrodes (13-15 $\text{M}\Omega$) containing 116 mM Kgluconate, 4 mM KCl, 10 mM HEPES, pH 7.2. Membrane patches were voltage-clamped with an Axopatch 200 amplifier (Axon Instruments). Continuous recordings were low-pass Bessel filtered at 1 kHz, acquired at 10 kHz using Pulse software (Heka Elektronik) and stored directly on a Macintosh Quadra 650. Single channel recordings were analyzed with MacTac (SKALAR Instruments) using the "50% threshold" technique to estimate event amplitudes and duration. and each transition was visually inspected before being accepted. Amplitude histograms were constructed using MacTacfit (SKALAR Instruments).

Only events lasting at least 1ms were included, and amplitude histograms were fitted by single Gaussian distributions. All experiments were performed at room temperature.

The expression of the hIK1 in *Xenopus* oocytes was readily detectable. Voltage ramp commands delivered to inside-out patches excised into 5 μM Ca^{2+} evoked robust, inwardly rectifying macroscopic current responses, not present in patches from uninjected oocytes (not shown) or inside-out patches bathed in Ca^{2+} -free media. Voltage step commands evoked large time-independent currents only when Ca^{2+} was included in the (bath) internal solution. Altering the external K^+ concentration (substituted by Na) shifted the reversal potential in accord with the Nernst prediction for a K^+ -selective conductance (57 mV/10-fold change in K^+). Similar to SK2 channels, currents evoked by voltage ramp commands were dependent upon the concentration of Ca^{2+} applied to the internal face of the membrane.

Example 4

Example 4 describes the calcium sensitivity of rSK2 and hSK1 channels.

Using inside-out micropatches as described above, rSK2 currents evoked by voltage ramps were shown to dependent upon the concentration of calcium in the internal (bath) solution. The slope conductance at the reversal potential was plotted as a function of calcium concentration and the data points fit with the Hill equation. From 8 patches, the average K_d for calcium was $0.63 \pm 0.23 \mu\text{M}$. The steep dependence upon calcium seen from the plot is reflected by a Hill coefficient of 4.81 ± 1.46 , suggesting that at least two calcium ions are involved in channel gating. Similar experiments performed with hSK1 yielded a K_d of $0.70 \pm 0.06 \mu\text{M}$ and a Hill coefficient of 3.90 ± 0.45 .

To compare hIK1 and SK2, normalized current was plotted as a function of Ca^{2+} concentration, and the data points fitted with the Hill equation. Both channels showed the same $K_{0.5}$ (concentration for half-maximal activation, $0.32 \pm 0.03 \mu\text{M}$ ($n=7$) for hIK1 and $0.31 \pm 0.05 \mu\text{M}$ ($n=4$) for SK2; $p=0.68$), but differed in the steepness of the Ca^{2+} -dependence; SK2 had a Hill coefficient of 3.5

± 0.4 ($n=4$), while hIK1 had a Hill coefficient of 1.7 ± 0.3 ($n=7$, $p<0.001$). These results demonstrate that hIK1 is also a calcium-activated potassium channel.

Example 5

Example 5 describes the magnesium induced inward rectification for the rSK2 channel.

The inward rectification for rSK2, described above, was observed in the absence of internal cations other than potassium and calcium ($5 \mu\text{M}$). Native SK channels exhibit inward rectification induced by internal Mg^{2+} ions (Lancaster et al., *J. Neurosci.*, 11:23-30 (1991)). In the hippocampus, SK channels exhibit significant inward rectification in the presence of internal Mg^{2+} (*Id.*). Currents were elicited from an inside-out macropatch excised from an oocyte expressing rSK2 in the presence of the varying concentrations of internal Mg^{2+} and $10 \mu\text{M}$ Ca^{2+} . When different concentrations of Mg^{2+} ($0.1\text{-}3 \text{ mM}$) were added to the solution bathing inside-out patches, outward currents were significantly reduced.

The concentration- and voltage-dependence of Mg^{2+} induced inward rectification was examined. A slight decrease of the inward current with increasing Mg^{2+} was observed. Therefore, the ratio of the outward current at potentials between 20 and 100 mV to the inward current at -100 mV was plotted as a function of the different concentrations of internal Mg^{2+} . From multiple experiments, the data points obtained at different Mg^{2+} concentrations and voltages were fit with the Hill equation, yielding an average Hill coefficient of 0.94 ± 0.27 ($n = 24$). Subsequently, the Hill coefficient was fixed at 1, and the mean K_d was plotted as a function of the test potential. The K_d decreased with increasing voltages suggesting that Mg^{2+} block was voltage-dependent. K_d for Mg^{2+} was obtained from 5 patches as shown in panel B at 20, 40, 60, 80 and 100 mV. Values at each potential were averaged, plotted as a function of voltage and fit with the Woodhull equation, $K_d(0\text{mV}) \exp(\delta z F E / RT)$ where the $K_d(0\text{mV}) = 6 \text{ mM}$, δ is the fraction of the electric field sensed by the Mg^{2+} ion, 0.30, z is the valence, 2, and F , E , R , and T have their usual meanings (Woodhull, *J. Gen. Physiol.*, 61:687-708 (1973)). Applying the Woodhull equation suggested that the Mg^{2+} ion senses approximately 0.30 of the membrane electric field.

Example 6

Example 6 describes single channel recordings from oocytes.

5 6A. Example 6A describes single channels were examined using inside-out patches excised from oocytes expressing rSK2. Addition of calcium at submicromolar concentrations induced channel activity not seen in controls. A representative patch showed that 0.2 μM calcium applied to the bath solution induced openings to a single amplitude. Channel activity increased as the calcium concentration was raised, such that in 0.6 μM calcium unitary openings could no longer be resolved. Upon washout of calcium, channel activity
10 disappeared. Channel activity in the presence of 0.4 μM calcium was recorded at several voltages. Similar to macroscopic ramp recordings, channel open probability was not obviously dependent upon voltage.

Unitary openings measured at several voltages were used to
construct a single channel I-V relationship. Solutions used were the same as for
15 macropatch recordings (Example 5). Electrodes were pulled from Corning 7052 glass (Garner) and had resistances of 9-13 M Ω . Data were filtered at 1 kHz (Bessel), acquired at 10 kHz using Pulse (HEKA Elektronik) and stored directly on a Macintosh Quadra 650. Single channels were analyzed using MacTac (SKALAR Instruments). The "50 % threshold" technique was used to estimate
20 event amplitudes. The threshold was adjusted for each opening and each transition was inspected visually before being accepted. Amplitude histograms were constructed using MacTacfit (SKALAR Instruments) and best fit by a single Gaussian distribution. Channel open probability was estimated as NP(o), the product of the open probability multiplied by the number of channels. NP(o) was
25 calculated as the sum of the (dwell time x level number) divided by the total time. N was estimated as the number of simultaneously open channels at 0.4 μM calcium. Linear regression analysis on three patches from an oocyte expressing either rSK2 or hSK1 yielded a mean single channel conductance of 9.9 ± 0.9 pS and 9.2 ± 0.3 pS, respectively.

6B. Example 6B describes the single channel conductance of hIK1. The methodology is described above in Example 3B. Stationary recordings from inside-out patches excised into a bathing solution containing 0.2-1.0 μM free calcium showed short-duration openings not seen in the absence of calcium. Representative traces were recorded at -60 mV. The degree of channel activity depended upon the concentration of internal calcium. Reducing intracellular calcium reduced channel activity, and removing internal calcium abolished channel activity, which returned after reapplication of Ca^{2+} . Sustained channel activity was seen at membrane voltages ranging from -100 mV to +100 mV and open probability was not obviously voltage-dependent. For select patches, the amplitudes of openings were measured, assembled into histograms, and fit by Gaussian distributions. The resulting mean amplitudes were used to construct the current-voltage relationship. The single channel current-voltage relationship shows inward rectification similar to the macroscopic current-voltage relationship. For this patch, linear regression analysis of the inward current-voltage relationship yielded a single channel conductance of 35 pS; results from four patches gave a unit conductance of 38 ± 4 pS. Measurements of the outward conductance were more variable, ranging from 5 to 12 pS.

Example 7

Example 7 describes the pharmacology of the novel rat and human potassium channels.

7A. Macroscopic rSK2 currents were recorded in 5 μM Ca^{2+} from inside-out macropatches with either 0 or 60 pM apamin or 0 or 2 μM d-tubocurare in the patch pipette described in Example 3. The functional characteristics of the cloned channels are reminiscent of the SK class of calcium-activated potassium channels described in neurons (Lancaster and Adams, *J. Neurophysiol.*, 55:1268-1282 (1986); Lancaster *et al.*, *J. Neurosci.*, 11:23-30 (1991); Sah *et al.*, *J. Neurophysiol.*, 68:1834-1841 (1992)), skeletal muscle (Blatz and Magleby, *Nature*, 323:718-720 (1986)), adrenal chromaffin cells (Park, *J. Physiol.*, 481:555-570 ((1994); Artalejo *et al.*, *Pflugers Archiv.*, 423:97-103 (1993)), and T-lymphocytes (Grissmer *et al.*, *J. Gen. Physiol.*, 99:63-84 (1992)). Native SK channels present

a distinct pharmacology. They are not blocked by the scorpion peptide, charybdotoxin (CTX), a potent blocker of BK potassium channels (Miller *et al.*, *Nature*, 313:316-318 (1985)). However, many but not all SK channels are blocked by the bee venom toxin, apamin, and the plant alkylid, d-tubocurare (dTC; Zhang and McBain, *J. Physiol.*, 488:661-672 (1995), Park, *J. Physiol.*, 481:555-570 (1994); Dun et al., *J. Physiol.*, 375:499-514 (1986)). Application of 500 nM CTX did not block rSK2 or hSK1, but abolished the activity of hSlo BK currents. rSK2 currents were potently blocked by picomolar concentrations of apamin with a K_d of 63 pM. In contrast, application of 100 nM apamin did not affect hSK-1 currents ($n = 8$). dTC also blocked rSK2 currents with a K_d of 2.4 μ M, while hSK1 was approximately 30-fold less sensitive, with a K_d of 76.2 μ M.

7B. For the pharmacology tests of hIK1, Clotrimazole was from Sigma, ketoconazole and iberiotoxin were from RPI, apamin was from Calbiochem, charybdotoxin was the generous gift of Dr. Chris Miller. The functional characteristics of hIK1 are reminiscent of intermediate conductance calcium-activated K^+ channels described from red blood cells (the Gardos channel; Gardos, 1958) and other tissues. Native IK channels present a distinguishing pharmacology, being blocked by charybdotoxin (CTX) but, different from large conductance voltage- and Ca^{2+} -activated K^+ channels (BK channels), are not blocked by iberiotoxin. Also, IK channels are not sensitive to the bee venom peptide toxin apamin, a blocker of certain native and cloned SK channels. In addition, some IK channels, notably the Gardos channel, are sensitive to several imidazole derivatives such as clotrimazole, but are not sensitive to others such as ketoconazole. hIK1 currents were potently blocked by CTX, with a K_i of 2.5 nM ($n = 4$), while 50 nM IBX blocked only $15 \pm 3 \%$. Human IK1 was sensitive to clotrimazole with a K_i of 24.8 nM, but was only $24 \pm 6 \%$ blocked by 10 μ M ketoconazole. 100 nM apamin reduced hIK1 currents by only $12 \pm 5 \%$.

All publications and patents mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication or patent was specifically and individually indicated to be incorporated herein by reference.